Factors Affecting Antibiotic Resistance of *Staphylococcus aureus*

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Dedicated to my parents and my boyfriend Diego

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Zusammenfassung

Staphylococcus aureus ist einer der weltweit häufigsten Erreger von nosokomialen und auch Spital unabhängigen Infektionen. *S. aureus* hat die Fähigkeit, Resistenzen gegen nahezu alle klinisch verwendeten Antibiotika zu entwickeln, wodurch die Behandlungsmöglichkeiten und der Behandlungserfolg einschränkt werden. Das Resistenzniveau und die intrinsische Toleranz von *S. aureus* gegen Antibiotika wird durch verschiede genomische Faktoren beeinflusst.

Einer dieser Faktoren ist das Zweikomponentensystem VraSR, welches die Stressantwort auf zellwand-aktive Antibiotika kontrolliert. Das Zweikomponentensystem reguliert Zellwand-Biosyntheseenzyme hoch und Autolysine runter, was einen intrinsischen Schutz von *S. aureus* gegen Zellwand-Antibiotika zur Folge hat. Im ersten Teil dieses Projektes wurde die Induktionskinetik vom VraSR-abhängigen Zellwand-Stress Stimulon von zehn Antibiotika mit Hilfe eines Luziferase-Reporterkonstrukts bestimmt. Hierbei wurde eine deutliche Korrelation zwischen der Induktionskapazität eines Antibiotikums und seinem bakteriziden Effekt aufgezeigt. Die Konzentrationsabhängigkeit der Induktion und die Lag-Phase waren für verschiedene Antibiotika variabel. Die Relevanz von VraSR für die Resistenz und die intrinsische Toleranz von verschiedenen Zellwand-Antibiotika wurde mit Hilfe einer VraR-Mutante validiert und liess eine speziell wichtige Rolle der von VraSR kontrollierten Stressantwort für den Schutz von *S. aureus* gegen Bacitracin, Flavomycin und Teicoplain erkennen.

Ein weiterer Faktor mit Einfluss auf die Methicillin-Resistenz von *S. aureus* ist die sogenannte LytR-CpsA-Psr (LCP) Proteinfamilie. In *Bacillus subtilis* wurde entdeckt, dass LCP Proteine die Ligation von Wandteichonsäuren an das Zellwand-Pepidogylcan katalysieren, den für lange Zeit unbekannten Schritt in der Biosynthese vom wichtigen Zellwandbestandteil Wandteichonsäuren. In diesem Projektteil konnte dieselbe Funktion indirekt für die drei LCP Proteine von *S. aureus* bestätigt werden mittels einer nahezu nicht mehr detektierbaren Menge von Wandteichonsäuren in der LCP Dreifachmutante. Der Zusammenhang von LCP Proteinen und der Biosynthese von Wandteichonsäuren wurde weiter erhärtet durch die partielle Komplementierung des Wachstumsdefektes der LCP Dreifachmutante durch Hemmung des ersten Biosyntheseschrittes von Wandteichonsäuren (TarO). Diese Hemmung von TarO verhindert wahrscheinlich die Akkumulierung von toxischen Zwischenprodukten und die Blockade des Lipidträgers, wodurch die nachteiligen Effekte der LCP Deletion teilweise aufgehoben werden. Weil die drei LCP Proteine zum VraSR-Regulon gehören, wurde ihre Verbindung zum Zellwand-Stress Stimulon untersucht. Die Deletion von LCP Genen führt zu einer starken Induktion der VraSR abhängigen Zellwand-Stressantwort; vergleichbar mit der bisher bekannten Induktion infolge der Limitierung von essentiellen Zellwand- oder Wandteichonsäuren-Biosyntheseenzymen und impliziert daher ebenfalls einen Zusammenhang von LCP Proteinen und der Biosynthese der Zellhülle.

Im dritten Teilprojekt wurde ein genetischer Faktor entdeckt, der die Methicillin-Resistenz und die Fitness von Methicillin-resistenten *S. aureus* (MRSA) beeinflusst. Die Sequenzierung des gesamten Genoms von zwei theoretisch genetisch identischen MRSA mit Unterschieden in der Wachstumsrate und der Methicillin-Resistenz zeigte nur einen Einzelnukleotid-Polymorphismus (SNP) im Diadenlyate Zyklase Gen *dacA* auf. DacA synthetisiert den kürzlich entdeckten sekundären Botenstoff c-di-AMP, ein Signalmolekül das zuvor schon mit der Zellwand-Homöostase in Verbindung gebracht wurde. Es konnte bestätigt werden, dass reduzierte zelluläre Mengen von c-di-AMP, infolge der Mutation in *dacA,* mit einem erniedrigten Beta-Lactam Resistenzniveau und mit einer erhöhten Wachstumsrate korrelieren.

Das vierte Teilprojekt untersuchte den Effekt einer Standardbehandlung mit Minocyclin, Amoxicillin oder Placebo auf die *S. aureus*-Population von gesunden Freiwilligen. Die *S. aureus*-Isolate wurden durch einen Partner des Intereuropäischen 7. Rahmenprogramm Projekts ‚ANTIRESDEV' gesammelt, der die klinische Studie durchführte. Die *S. aureus*-Isolate wurden im Detail analysiert in Bezug auf Antibiotikaresistenz-Profile, Fitness, MLST-, PFGE- and *spa*-Typen. Die Kombination der Fitnessdaten und des Resistoms zeigte keine offensichtliche Korrelation zwischen der Fitness und der Präsenz oder Abwesenheit von Resistenzgenen oder des Resistenzniveaus. Die Freiwilligen waren im Allgemeinen nur von einem spezifischen *S. aureus*-Stamm besiedelt. Interessante Isolate von einem Freiwilligen mit drei verschiedenen Stämmen wurden noch detaillierter analysiert. Die Resultate der Studie suggerieren, dass sich aufgrund einer Standardbehandlung mit Amoxicillin oder Minocyclin keine Resistenzen oder Anreicherungen von Resistenzgenen in den kommensalen *S. aureus* entwickeln.

Summary

Staphylococcus aureus is one of the major causes of both nosocomial and community acquired infections worldwide. *S. aureus* is able to acquire resistance to virtually all antibiotics prescribed limiting treatment options and impairing treatment outcomes. Resistance levels or intrinsic tolerance to antibiotics is influenced by various genetic factors of *S. aureus*.

One of these factors is the two-component system VraSR that controls a stress response to cell wall-active antibiotics. The VraSR two-component system up-regulates cell wall biosynthesis enzymes and down-regulates autolysins resulting in an intrinsic protection of *S. aureus* against cell wall antibiotics. In a first part of this project induction kinetics of the VraSR-dependent cell wall stress stimulon in response to ten antibiotics were determined using a luciferase reporter construct. A clear connection between the induction capacity of an antibiotic and its bactericidal effect was shown. The concentration dependency of induction and lag phases were found to be variable for different antibiotics. The importance of VraSR for resistance or intrinsic tolerance to different cell wall active antibiotics was validated using a VraR mutant strain revealing that the VraSR controlled stress response is especially important in protection of *S. aureus* against bacitracin, flavomycin and teicoplanin.

Another factor influencing methicillin resistance of *S. aureus* is the so-called LytR-CpsA-Psr (LCP) protein family. LCP proteins were suggested to catalyse the long missing step of ligation of the important cell wall component wall teichoic acids to the cell wall peptidoglycan in *Bacillus subtilis*. This was indirectly confirmed for the three *S. aureus* LCP proteins by showing that the amount of wall teichoic acids was almost abolished in a *S. aureus* triple LCP mutant in this part of the project. The connection of LCP proteins to biosynthesis of wall teichoic acids was also substantiated by partial complementation of the growth defects of the LCP triple mutant by inhibiting the first step of WTA synthesis (TarO). The inhibition of TarO probably limits accumulation of toxic intermediates or blockage of the lipid carrier, partially rescuing the detrimental effect of LCP deletion. Since the three LCP genes belong to the VraSR regulon, the connection to the cell wall stress stimulon was analysed. Deletion of LCP genes highly induced the VraSR system similar to the depletion of essential cell wall or wall teichoic acid biosynthesis enzymes observed previously, providing thereby another confirmation for a connection of LCP proteins to cell envelope biosynthesis.

The third subproject identified a genetic factor that influences both methicillin resistance and fitness of MRSA. Whole genome sequencing of two theoretically genetically identical MRSA with different fitness and resistance characteristics revealed only one single nucleotide polymorphism (SNP) in the diadenlyate cyclase gene *dacA*. DacA synthesises the recently discovered second messenger c-di-AMP, a signalling molecule that was before connected to cell wall homeostasis. It was confirmed that the

reduced cellular level of c-di-AMP caused by the *dacA* mutation correlates with a decrease in beta-lactam resistance levels and increases in the growth rate.

The fourth sub-project analysed the effect of a standard therapy with minocycline, amoxicillin or placebo on the *S. aureus* population of healthy volunteers. The *S. aureus* isolates were collected by a partner of the Intraeuropean 7th framework project 'ANTIRESDEV' who performed the clinical study. The *S. aureus* isolates were analysed in detail with respect to their antibiotic resistance profile, fitness, MLST, PFGE and *spa* types. The combination of the fitness data and the resistome showed no apparent correlation between the fitness and the presence or absence of resistance genes, or the resistance levels. Volunteers carried generally only one specific *S. aureus* strain. Interesting isolates from one volunteer who carried three different strains were analysed in more detail. The results of the study suggest that there is no resistance development or accumulation of resistance in commensal *S. aureus* strains upon a standard treatment with amoxicillin or minocycline.

1 Introduction

1. 1 Staphylococcus aureus – profile of a successful pathogen

1. 1. 1 Taxonomy, morphology and general biology

Staphylococcus aureus is a Gram-positive non-spore forming and non-motile bacterium that belongs to the family of Gram-positive cocci, Staphlyococcaceae, in the order of Bacillales in the phylum Firmicutes. *S. aureus* is a spherical bacterium that forms grape like cell aggregates and often yellow/golden colonies (Figure 1), three characteristics that led to its name. *Staphyl* originates from the Greek word '*staphylé'* for a bunch of grapes indicating the cell arrangement, *coccus* originate from the Greek word '*kókkos*' means berry or grain indicating the cell shape and *aureus* comes from '*aureus*' meaning golden in Latin for the yellow/golden pigmentation of the colonies. The pigmentation is caused by carotenoids that protect the bacterium against oxidants and are therefore also considered as virulence factors (see section 1.1.3, [1]). The spherical *S. aureus* cells have a diameter of approximately 0.7-1.2 µm (Figure 1). The *S. aureus* cell is composed of cytoplasm surrounded by the cell membrane, a thin periplasmic space and a thick cell wall, and for some strains a polysaccharide capsule [2,3]. The cell wall is composed of a thick peptidoglycan layer (see section 1.2.1) that is decorated by covalently attached wall teichoic acids (see section 1.4) and drawn through by lipoteichoic acids anchored in the cytoplasmic membrane [4,5]. Another component connected to the peptidoglycan layer are cell surface proteins including adhesive proteins such as protein A, fibronectin binding protein or clumping factor A (see section 1.1.3, [6]). The capsule of *S. aureus* is characterised by typing in eleven microcapsular polysaccharide serotypes of which type 5 and type 8 are most commonly found in clinical isolates [7]. The main function of the cell envelope is to protect the bacteria from its high tugor pressure and maintains the cell shape [8].

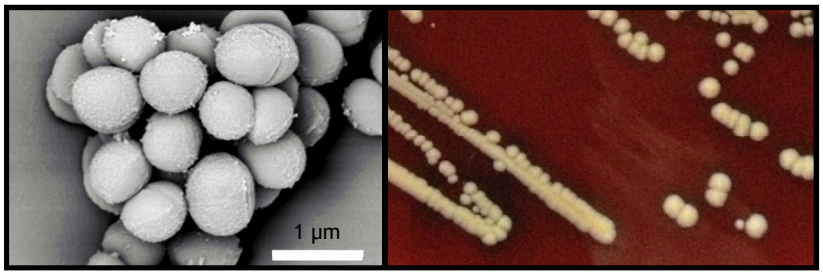

Figure 1: Scanning electron microscopy of *S. aureus* [9] and *S. aureus* colonies on sheep blood agar.

S. aureus is a facultative anaerobe that is able to grow by both generating energy by aerobic respiration or by fermentation yielding mainly in lactic acid formation. A biochemical characteristic that allows differentiation of Staphylococci from other Gram-positive cocci, like Streptococci, is their capability to hydrolyse hydrogen peroxide classifying them as catalase-positive. *S. aureus* produce coagulases that differentiate them from other coagulase-negative Staphylococci species such as

Staphylococcus epidermidis. Both catalase and coagulase are virulence factors as described below in section 1.3.3. Differentiation of strains within the species of *S. aureus* for epidemiological purpose is mainly done by three state-of-the-art methods: *i)* multi locus sequence typing (MLST) that types strains by sequencing and assigning of alleles of seven housekeeping genes (carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*)) to different sequence types [10]; *ii) spa* typing differentiates strains by sequencing of the hyper variable part of the gene encoding protein A [11,12]; *iii)* pulsed field gel electrophoresis (PFGE) of *SmaI* digested chromosomal DNA results in strain specific band patterns of about ten to twenty bands of different sizes and the method is still considered the gold standard for differentiating *S. aureus* strains [13,14]. Other less commonly used typing methods include coagulase typing [15], *agr* typing [16,17], typing of the staphylococcus cassette chromosome *mec* (SCC*mec*) element (see section 1.2.2.2, [18,19]) and the detection of present or absence of certain toxins such as Panton-Valentine leukocidin (PVL) and the toxic shock syndrome toxin (TSST-1) [20].

1. 1. 2 Prevalence and infections

S. aureus is part of the human normal flora predominantly of the anterior nares and of the skin. On average 20% of human population are persistent *S. aureus* carriers that are always colonised and 60% of the population carry *S. aureus* transiently, called intermediate carriers [21]. *S. aureus* is an opportunistic pathogen and colonisation is generally harmless for healthy individuals; nevertheless, *S. aureus* carriage increases the risk for *S. aureus* infections [22]. The reservoir of *S. aureus* on the skin and in the nose can become problematic if the natural defence is impaired by immune deficiency, injuries of the skin or implantation of biomedical devices that may result in people getting infected by either their otherwise commensal *S. aureus* strain or from an external *S. aureus* source [21]. Certain endemic and especially virulent strains are also reported to cause infections in healthy individuals with no obvious risk factors [23].

S. aureus is besides being part of the normal flora a dangerous pathogen and a major cause of both community-acquired and nosocomial infections. Nosocomial infections include skin and soft tissue infections, such as bullous impetigo, abscesses, furunculosis and staphylococcal scaled skin syndrome, but also more severe infections like catheter-associated bacteraemia, ventilator-assisted pneumonia or deep-seated infections like meningitis, endocarditis or osteomyelitis. A recent national wide study in Switzerland identified *S. aureus* as the most frequent cause of surgical site infections [24]. Community-acquired infections are mainly skin and soft tissue infections and infections of the respiratory tract. This infection can become severe or even life-threatening when caused by especially virulent strains resulting in necrotizing fasciitis or necrotizing pneumonia [25]. Today, the majority of community associated infections are caused by endemic methicillin resistant strains such as USA300 or EMRSA-16 as also discussed in section 1.2.2.

1. 1. 3 Virulence factors

S. aureus is well equipped with a whole arsenal of virulence factors allowing them to infect different tissues and to evade immune defence mechanisms (Figure 2, [6]). The combination of virulence factors a strain possesses and their expression levels defines the strains pathogenic potential. However, since *S. aureus* is an opportunistic pathogen, the success is also strongly dependent on the host conditions [26].

To cause an infection a pathogen must in a first step be able to attach to host cells. *S. aureus* contain many different adhesins that enable attachment to host cells, either directly by surface factors classified as 'microbial surface components recognising adhesive matrix molecules' (MSCRAMMs) [27] or indirectly by secreted factors summarized as 'secretable expanded repertoire adhesive molecules' (SERAM) [28]. These factors include among other fibronectin binding proteins (FnbpA and FnbpA), collagen binding proteins (Cna), clumping factors (ClfA and ClfB), the plasma-sensitive surface protein (Pls), protein A (SpA), coagulase (Coa) and the extracellular adhesive protein (Epa). Besides attachment to host tissues and surfaces of biomedical devices, about 45% to 70% of *S. aureus* strains are able to form biofilms that protects the bacteria from the host immune system and antibiotics [29].

In a next step, the bacteria must be able to invade the host tissue and to acquire nutrients. *S. aureus* secrete various proteins or exotoxins that allow lysis of host cells such as the haemolytic toxins, α-haemolysin, β-haemolysin, δ-toxin, γ-haemolysin and Panton-Valentine leukocidin (PVL), proteases, lipases, hyaluronidases and nucleases [23,25].

In parallel, the bacteria need to persist against the host immune system. An overview of the *S. aureus* virulence factors with a special focus on immune evasion is shown in Figure 2. *S. aureus* can inhibit the immune response at different steps. The recruitment of phagocytes is inhibited by the chemotaxis inhibitory protein of *S. aureus* (CHIPS) that binds to the leukocyte receptors C5a and N-formyl peptides blocking thereby the receptors of chemoattractants [30]. Phagocytes can be lysed by pore-forming toxins like γ-haemolysin or PVL. The complement system is inhibited by the extracellular fibrinogen-binding protein (Efb) that binds to the complement component C3 and by the staphylococcal complement inhibitory protein (SCIN) that inhibits C3 convertases [31,32]. Carotenoid pigments and the catalase protect from the effectors of immune cells by providing antioxidant shield or detoxifying hydrogen peroxide, respectively [1]. Protein A prevents recognition of *S. aureus* by phagocytes by decorating the *S. aureus* surface with host antibodies through binding of the Fc domain of the immunoglobulins. Opsonisation is also limited by the polysaccharide capsule by the fibrinogen binding clumping factor and by poly-*N*-acetylglucosamine (PNAG) [33,34]. The staphylokinase (SAK) can activate the conversion of plasminogen (PG) to active plasmin that degrades the opsonin of the

complement system (C3b) and human immunoglobulin G (IgG) [35]. Positive charge modification of the cell envelope, by D-alanylation of teichoic acids (*dlt* operon) or transfer of L-lysine to membrane lipid phosphatidylglycerol resulting in lysyl-phosphatidylglycerol (MprF), protects from human cationic antimicrobial peptides [36-38].

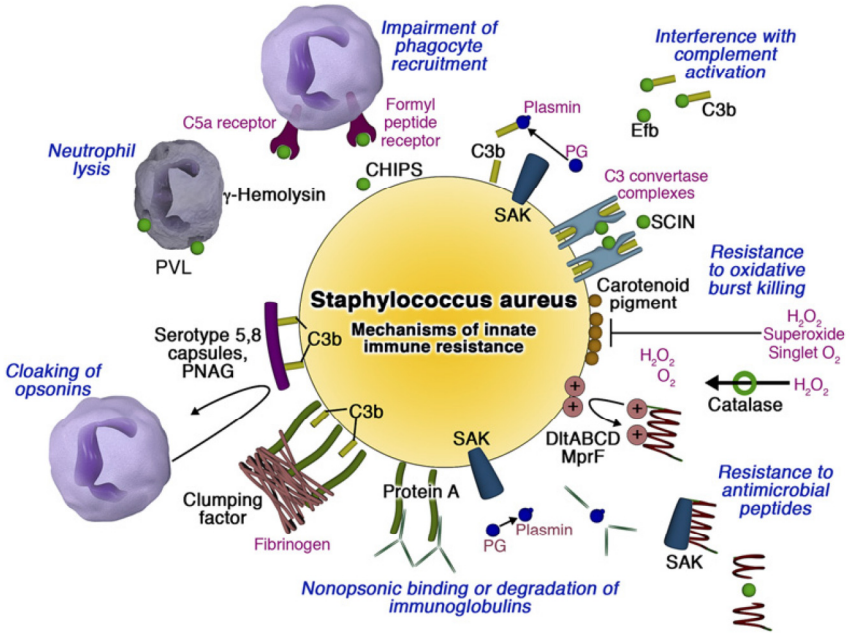

Figure 2: Overview of *S. aureus* virulence factors with focus on the mechanisms of innate immune resistance. Abbreviations are explained in the main text. Adapted from [6].

Another strategy of *S. aureus* to overcome the immune system is hyper activation of the immune system through superantigens. The T-cell response is excessively und unspecificially activated by binding of superantigens directly to MHC class II molecule and the T-cell receptor. The resulting activation of an abnormally high number of T-cells leads to massive release of proinflammatory cytokines and recruitment of B- and T- cells to the site of infection that can lead to a shock [39]. Superantigens are the toxic shock syndrome toxins (TSST) or enterotoxins (SEA-SEH) that are causing agents of the toxin mediated diseases toxic shock syndrome and food poisoning, respectively [40].

1. 1. 4 Genome and mobile genetic elements

The genome of *S. aureus* is composed of one circular chromosome that has a size of about 2.8 Mb and a low GC content of approximately 33%. The first complete genome sequences of the strains Mu50 and N315 were published in 2001 [41]. To date, complete genome sequences for 43 *S. aureus* strains are available in the NCBI Genome database. Much more data is accessible as incomplete genome scaffolds or contigs from next generation sequencing projects of hundreds of strains. The genome of *S. aureus* contains about 2700 protein encoding genes plus structural and regulator RNAs. Of these genes about 75% are classified as core genome conserved in the vast majority of the strains (>95%)

[3]. The core genome is mainly composed of genes encoding essential metabolic and regulatory proteins as well as surface proteins. The remaining 25% is called accessory genome, genetic material that is dispensable and only present in about 50% of the strains. This includes mobile genetic elements, such as pathogenicity islands, genomic islands, prophages, transposons, insertion sequences and chromosomal cassettes like the SCC*mec* element ([3,42], see section 1.2.2.2). Besides the chromosome, *S. aureus* can also carry genetic information on plasmids that is also counted to the accessory genome [42].

1. 2 Antibiotics and antibiotic resistance in S. aureus

S. aureus is able to rapidly adapt to changes in the environment including exposure to antibiotics and it is able to develop resistance to virtually all antibiotics. Especially, multi-resistant strains are limiting treatment options resulting in impaired treatment outcomes. There are three general principles by which a bacterium can become resistant to antibiotics. First, the antibiotic can be inactivated or modified; second, the target of the antibiotic can be altered; and third, the accumulation of the antibiotic can be prevented by efflux or uptake inhibition. These three principles can be achieved by mutation in the chromosome, accumulation of additional genetic material or by up-regulation or down-regulation of certain pathways [43].

Antibiotic targets need to be enzymes or pathways that are unique to prokaryotes to prevent cytotoxicity for human cells and to limit side effects. The first antibiotic penicillin, accidentally discovered by Alexander Fleming in 1928, inhibits cell wall synthesis [44]. The discovery of penicillin provided the basis for the development of the diverse class of very effective beta-lactam antibiotics including carbapenems and cephalosporins and it is still the most frequently used class of antibiotics.

1. 2. 1 Cell wall biosynthesis as an antibiotic target

The cell wall of *S. aureus* is made of peptidoglycan (also called murein), a heteropolymer that consists of alternating *N*-actetyl muramic acid (MurNAc) and *N*-acetyl glucosamine (GlcNAc) connected by short peptide chains [5]. Omitting the biosynthesis of precursors, the first step of peptidoglycan synthesis is the addition of phosphoenolpyruvate (PEP) to uridine diphosphate (UDP) GlcNAc catalysed by the UDP-*N*-acetyl-glucosamine-3-enolpyruvyl transferase MurA resulting in UDP-*N*-acetylenolpyruvyl-glucosamine (UDP-GlcNAcEP) as shown in Figure 3. Fosfomycin inhibits this very first step by covalent modification of the enzyme MurA [45]. MurB then catalyses NADPH-dependent reduction of UDP-GlcNAcEP to the UDP-MurNAc followed by attachment of an L-alanine, a D-glutamate and an L-lysine to UDP-MurNAc by the enzymes MurC, MurD and MurE, respectively (Figure 3, [46]). D-cycloserine inhibits the next step, the addition of two D-alanines to the peptidoglycan precursor by inhibiting both the alanine racemase and the D-alanine:D-alanine ligase A

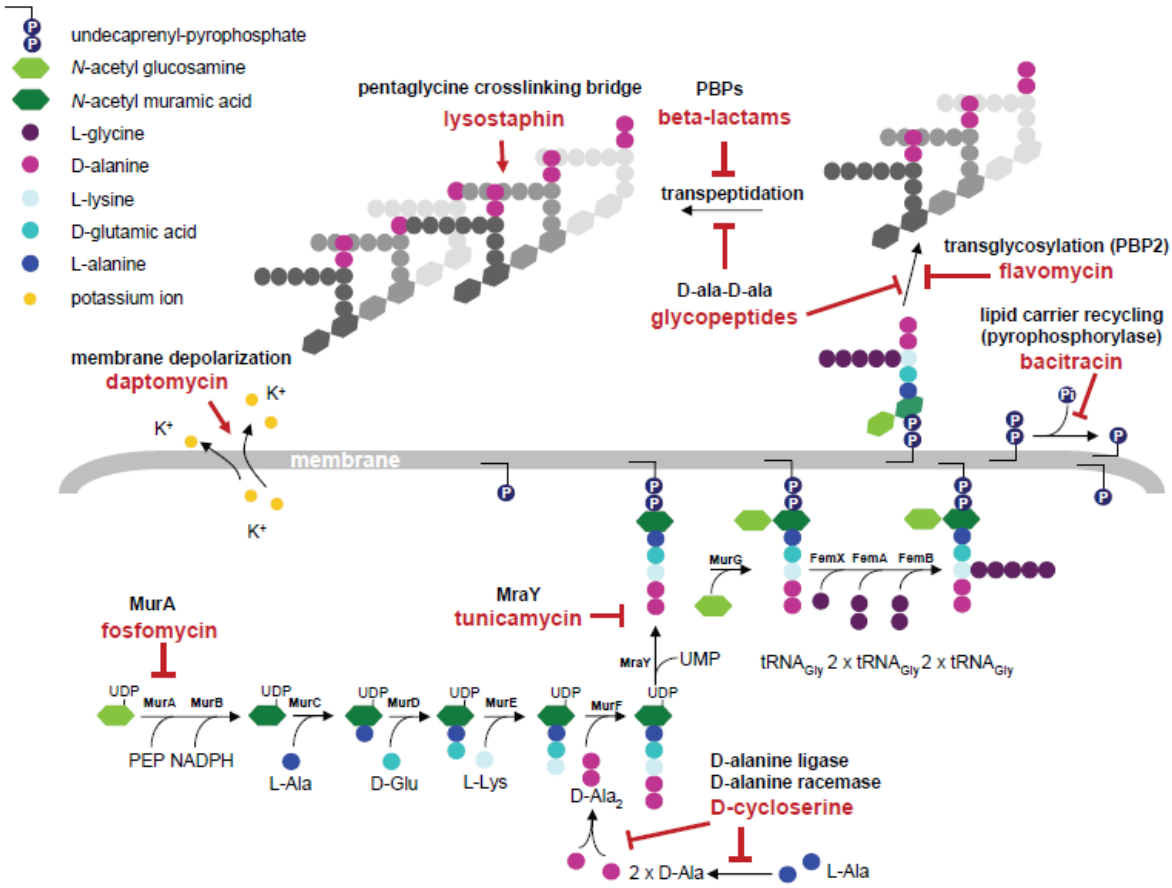

Figure 3: Schematic representation of the enzymatic steps involved in *S. aureus* cell wall synthesis and the targets of cell wall active antibiotics. Inhibition of enzymatic steps is indicated by blocked arrows, cleavage of the pentaglycine bridge by lysostaphin and disruption/depolarization of the membrane by daptomycin is indicated with arrows. Adapted from [46,47].

[47]. In the following step, the peptidoglycan precursor is attached to the undecaprenyl-pyrophosphate lipid anchor (also called C55-isoprenyl pyrophosphate or bactoprenol) by the transferase MraY resulting in the intermediate lipid I. This transfer by MraY is inhibited by tunicamycin [48,49]. Tunicamycin inhibits also the first enzyme of wall teichoic acid biosynthesis, the attachment of an UDP-GlcNAc to the undecaprenyl-pyrophosphate lipid carrier by TarO, even with a higher affinity (see section 1.4, [50]). Next, the glycosyl transferase MurG attaches an UDP-GlcNAc to the lipid bound precursor resulting in the intermediate lipid II. In the following, the pentaglycin bridge is formed by the non-ribosomal peptidyltransferases FemX, FemA and FemB resulting in the completed lipid anchored peptidoglycan monomer [51-53]. This final lipid II is flipped to the outside of the cell by a yet unknown enzyme [5]. Bacitracin inhibits cell wall biosynthesis by forming a metal dependent complex with the lipid carrier undecaprenyl-pyrophosphate and thereby preventing dephosphorylation of undecaprenyl-pyrophosphate, the recycling of the lipid carrier of cell wall synthesis [54,55]. The first step of the polymerisation of the precursors is transglycosylation by the bifunctional penicillin-binding protein 2 (PBP2). Transglycosylation is inhibited by flavomycin (also called moenomycin) through binding of the transglycosylase domain of PBP2 and thereby preventing the incorporation of the peptidoglycan subunits into the peptidoglycan matrix. Glycopeptide antibiotics such as

vancomycin or teicoplanin bind to the D-ala-D-ala of the lipid II shielding the substrate of transglycosylation and transpeptidation [56]. The final cross-linking of the peptidoglycan subunits by transpeptidation is catalysed by the four PBPs (PBP1-4). Tanspeptidation is inhibited by the above mentioned beta-lactam antibiotics such as oxacillin and penicillin that bind the transpeptidase active domain of penicillin-binding proteins (PBPs) [57]. Different beta-lactam antibiotics were detected to inhibit all PBPs, like ampicillin and oxacillin or specifically inhibit one PBP such as imipenem and meropenem (PBP1 selective), cefotaxime and ceftriaxone (PBP2 selective), cefaclor (PBP3 selective) and cefoxitin (PBP4 selective) [58-62].

Even though daptomycin acts primarily on the membrane it was also suggested to indirectly inhibit peptidoglycan synthesis and to act synergistically with PBP1 inhibiting beta-lactam antibiotics [58,63]. The current model of the mode of action proposes that daptomycin is delivered to the membrane in form of micelles that possibly further oligomerise leading to potassium efflux and cell death [64-66]. Besides antibiotics, also the zinc metalloenzyme lysostaphin from *Staphylococcus simulans* targets specifically the cell wall peptidoglycan of *S. aureus* by cleaving the pentaglycine cross-linking bridge [67].

1. 2. 2 Methicillin resistant *S. aureus*

The discovery of penicillin and the establishment of penicillin as agent for treatment of infections during the Word War II resulted in a drastic decrease of the lethality of *S. aureus* infections and bacterial infections in general. However, soon after the establishment of penicillin treatment, penicillin resistant *S. aureus* strains emerged limiting the effectiveness of penicillin treatment [68]. Penicillin resistant *S. aureus* have acquired a usually plasmid encoded beta-lactamase that cleaves the lactam ring of penicillin; and already towards the end of the 1950s 80% of *S. aureus* were resistant to penicillin [69]. Therefore, the semi-synthetic penicillinase resistant beta-lactam methicillin was developed and approved for treatment in 1959. Today, the more stable methicillin analogue oxacillin or other beta-lactam antibiotics are used in clinics. Within one year of the introduction of methicillin, the first methicillin resistant *S. aureus* (MRSA) were reported [70]. MRSA have acquired an additional penicillin-binding protein PBP2a that allows cell wall synthesis in presence of methicillin (see section 1.2.2.2). Infections with MRSA are problematic and difficult to treat, in particular, if the MRSA strains have acquired resistance to other antibiotic classes resulting in multi-drug resistant strains (see sections 1.2.2.2). The treatment of infections with last resource antibiotics is not only cost intensive for the health care system but it is also often less efficient, accompanied by severe side effects and it drives the selection for new resistances [71].

1. 2. 2. 1 Epidemiology of MRSA

The number of infections caused by methicillin resistant *S. aureus* (MRSA) is still increasing. A national wide study of the USA in 2010 detected that on average 41 in 1000 hospitalized patients were

colonised and 25 in 1000 hospitalized patients were infected with MRSA [72]. The overall MRSA incidences including both infection and colonisation in hospitals increased since 2006 from 46 in 1000 patients to 66 in 1000 patients in 2010 [72]. However, the number for invasive life-threatening MRSA infections declined from 2005 to 2008 in the USA [73]. The proportion of MRSA given relative to the total *S. aureus* incidences in the USA was 66.4% in 2009 [74]. In Europe, the situation is better with lower percentage of MRSA in most countries. However, MRSA rates differ a lot between geographic areas with an increase from north to south and rates can reach over 50% of the total *S. aureus* infection in certain countries as reported by European Center for Disease Prevention and control (ECDC) in 2011 (Figure 4). The reasons for the higher percentage of MRSA in southern countries are not completely understood. However, a correlation to the higher number of antibiotic prescriptions and less efficient precautions and interventions, for example screening and isolation of patients colonized with MRSA in hospitals, is suggested [75,76]. The percentage of MRSA infection in Switzerland was 10.2% in 2012 (Swiss Centre for Antibiotic Resistance, www.anresis.ch), comparable to the northern neighbouring countries. The emergence of so-called community-associated (CA-) MRSA over the last two decades clearly contributed to the increase of MRSA numbers. In the USA 45% of all MRSA were classified as CA-MRSA strains in 2009, such as the typical endemic strains CA-MRSA USA300 (see section 1.2.2.3, [74]). Interestingly, almost all MRSA clones worldwide belong to only five clonal complexes (CCs): CC5, CC8, CC22, CC30 and CC45 [23]; a clearly limited diversity compared to methicillin sensitive *S. aureus* (MSSA) that are more heterogeneous [69].

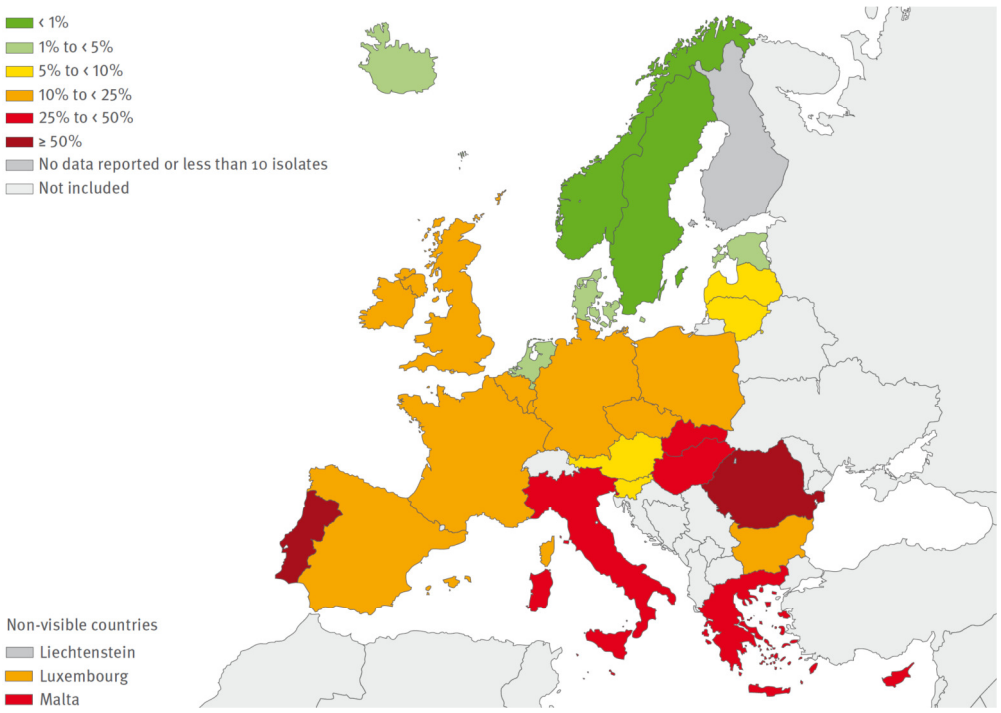

Figure 4: Prevalence of MRSA infections in Europe according to the European Centre for Disease Prevention and Control in percentage (%) of MRSA relative to the total number of *S. aureus* infections in 2011. Figure from www.ecdc.europa.eu.

1. 2. 2. 2 The staphylococcal cassette chromosome mec

Methicillin resistance is encoded on a mobile genetic element named staphylococcal cassette chromosome *mec* (SCC*mec*). The actual resistance gene m*ecA* encodes an alternative penicillin-binding protein PBP2a with lower affinity for beta-lactams than the endogenous PBPs, allowing cell wall synthesis in presence of beta-lactam antibiotics. The size of SCC*mec* elements varies between 21 kb to 69 kb, though larger mosaic SCC*mec* elements exist. SCC*mec* elements consist of the *mec* gene complex, the cassette chromosome recombinase (*crr*) gene complex and so-called J region (joining or junkyard regions), defined as regions other than the *mec* or *ccr* complex [19]. The composition of the *mec* gene complex varies and a complete complex is made of the resistance gene *mecA*, the repressor *mecI*, the signal transducer *mecR1*, the antirepressor *mecR2* (see section 1.2.2.4) and different insertion sequences (IS). The *mec* gene complex is according to its composition assigned to five different classes A, B, C1, C2 and E (Table 1, [18,19,77]). The presence or absence of the recently discovered *mecR2* has not been considered for SCC*mec* typing up to now. *mecR2* is generally present in the SCC*mec* types containing functional *mecR1-mecI* genes (SCC*mec* types II, III, VIII and XI) and absent in the other types [77]. The *ccr* gene complex allows the site specific integration of the SCC*mec* element into the *S. aureus* chromosome at the attachment site located at the 3' end of *orfX* (encoding a ribosomal methyltransferase of the RlmH type, [78]). There are eight types of *ccr* gene complexes composed of combinations of three phylogenetically distinct *ccr* genes with less than 50% sequence similarity (*ccrA*, *ccrB* and *ccrC*) and of subtypes of *ccrA* and *ccrB* with less than 85% nucleotide identity. So far, there are eleven SCC*mec* types classified according to the type of the *crr* complex and the *mec* gene complex (Table 1).

Table 1: SCC*mec* types of *S. aureus.*

SCC*mec* types	*ccr* gene complexes	*mec* gene complexes	strains examples
I	1 (A1B1)	B (*IS431-mecA-ΔmecR1-IS1272*)	NCTC10442, COL
II	2 (A2B2)	A (*IS431-mecA-mecR1-mecI*)	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	3 (A3B3)	A (*IS431-mecA-mecR1-mecI*)	85/2082
IV	2 (A2B2)	B (*IS431-mecA-ΔmecR1-IS1272*)	MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, EMRSA-15, JCSC6668, JCSC6670, USA300
V	5 (C1)	C2* (*IS431-mecA-ΔmecR1-IS431*)	WIS (WBG8318), TSGH17, PM1,
VI	4 (A4B4)	B (*IS431-mecA-ΔmecR1-IS1272*)	HDE288
VII	5 (C1)	C1 (*IS431-mecA-ΔmecR1-IS431*)	JCSC6082
VIII	4 (A4B4)	A (*IS431-mecA-mecR1-mecI*)	C10682, BK20781
IX	1 (A1B1)	C2* (*IS431-mecA-ΔmecR1-IS431*)	JCSC6943
X	7 (A1B6)	C1 (*IS431-mecA-ΔmecR1-IS431*)	JCSC6945
XI	8 (A1B3)	E (*blaZ-mecA*$_{LGA251}$-*mecR1*$_{LGA251}$-*mecI*$_{LGA251}$)	LGA251

* The two IS431 are arranged in opposite direction. Table adapted from www.sccmec.org and [18].

In addition to methicillin resistance, the SCC*mec* is a hotspot for integration of plasmids and transposons encoding genes conferring resistance for example to kanamycin, erythromycin and clindamycin, tetracycline, mercury (SCC*Hg* or SCC*mercury*) and cadmium [19,69,79]. SCC*mec*

elements can be linked to genes such as the arginine catabolic mobile element (ACME) and peptide toxin genes (PSM-*mec*) [18].

1. 2. 2. 3 Hospital-associated versus community-acquired MRSA

MRSA strains were for a long time restricted to hospitals setting or other areas with high antibiotic pressure. This so-called hospital-associated (HA-) MRSA strains carry often the early identified large SCC*mec* element of type I, II and III (see section 1.2.2.2) that have been acquired only by a limited number of clones [69,80]. Initially, MRSA showed a heterogeneous resistance profile, where only a small subpopulation can survive high beta-lactam concentrations. Over time, however, highly homogenously resistant strains evolved that were spreading throughout the hospitals of various countries from the 1970 onwards. Examples of early endemic MRSA clones are the Iberian clone (ST247, SCC*mec* type I), the Brazilian/Hungarian clone (ST239, SCC*mec* type III), the New York/Japan clone (ST5, SCC*mec* type II) and EMRSA 16 (ST32, SCC*mec* type II) (reviewed in [80,81]). These highly resistant HA-MRSA strains have often a reduced growth rate compared to MSSA strain that could be one explanation for their restriction to areas with high antibiotic pressure [82-85]. The reasons for the decreased fitness and competitiveness are suggested to result from the high methicillin resistance level, the maintenance of about 50 kb additional genetic material and from additional resistance determinants like tetracycline, kanamycin, erythromycin and clindamycin genes resulting in multi-drug resistant phenotype.

Over the last two decades, MRSA started to emerge into the community. These so-called CA-MRSA showed increased fitness and virulence leading to infections in individuals without obvious risk factors [23]. The methicillin resistance level of CA-MRSA is generally lower compared to HA-MRSA strains and CA-MRSA strains often show a heterogeneous resistance profile that nonetheless normally results in beta-lactam treatment failures [23,25]. CA-MRSA strains mainly carry smaller more recently discovered SCC*mec* elements, predominantly type IV but also type V. The decreased size of these elements and the general absence of additional resistance genes are suggested to reduce fitness costs and thereby possibly increase fitness of these strains compared to HA-MRSA [83,86]. It was also suggested that the AMCE element carrying an arginine deiminase gene often associated with SCC*mec* IV could increase fitness of the strains [86]. However, the exact reasons and mechanisms responsible for the differences in fitness and methicillin resistance of HA-MRSA and CA-MRSA remain unclear.

Increased virulence of CA-MRSA was for a long time connected to the presence of a highly potent cytotoxcin, the Panton-Valentine leukocidin (PVL), which is also used to differentiate between HA-MRSA and CA-MRSA. PVL is a two-component pore forming toxin that is immuno-resistant, cytotoxic to monocytes and polymorphonuclear neutrophils and encoded in the genes *lukS-PV* and *lukF-PV* located on different prophages [87,88]. The relevance of PVL for the increased virulence of CA-MRSA strains is still debated and CA-MRSA strains lacking PVL can also cause severe infections

and become endemic as for example observed for the Australian CA-MRSA [89]. Neutrophils from different animals were recently discovered to vary in their sensitivity to PVL. PVL is more active against human and rabbit neutrophils and less leukocidal for murine, rat or simian neutrophils explaining the confusing and contradictory results for PVL importance in animal infection models [90]. A current opinion from a systematic literature review is that PVL increases virulence of strains in skin and soft tissue infections but has only minor influence on pneumonia, musculoskeletal infections and bacteraemia [91]. Though, the importance of PVL in pneumonia is still highly debated today [92,93].

Another explanation for the increased virulence of CA-MRSA is the elevated expression of virulence factors of the core genome like α-toxin or so-called phenol-soluble-modulins (PSM) [25,94]. PSM are very potent against human neutrophils and are released at higher concentrations from CA-MRSA than from HA-MRSA and are therefore suggested to be a key element in increased virulence of CA-MRSA [95].

Since a few years, CA-MRSA emerge into hospital settings and especially endemic CA-MRSA like USA300 are causing increased incidences of hospital onset CA-MRSA infections [96]. In Europe, USA300 is also detected in hospitals but the most frequently observed CA-MRSA belong to ST80 and *spa* type t044 [71]. Nosocomial infections by CA-MRSA limit the original epidemiological differentiation and a genotype based definition was suggested for HA-MRSA and CA-MRSA [97].

1. 2. 2. 4 Factors influencing the methicillin resistance level

The methicillin resistance is dependent on regulation of the resistance determinant *mecA*. Initially, regulation of *mecA* expression was claimed to be controlled by the two-component system composed of *mecR1-mecI*, encoding a sensor-inducer and a repressor, respectively [98]. Recently, a third component, the anti-repressor MecR2, was discovered to be involved in *mecA* regulation and a model for three-component regulation is suggested [77]. Beta-lactam antibiotics bind to the extracellular sensor domain of MecR1 that leads to autocatalytic cleavage resulting in activation of the cytoplasmatic inducer domain of MecR1. The activated inducer domain of MecR1 directly or indirectly cleaves the dimers of the repressor MecI allowing transcription of *mecA* and of the *mecR1-mecI-mecR2* operon. MecR2 interacts with MecI and promotes the proteolytic cleavage of MecI that is essential for the *mecA* induction. In absence of beta-lactams MecI dimers are bound to the *mecA* promoters repressing transcription. Due to the structural and functional similarity of MecI to the repressor BlaI of the beta-lactamase gene *blaZ*, both repressors can bind to the *mecR1-mecI-mecR2* and *blaZ-blaR1-blaI* promoter regions [99]. It is also suggested that the presence of *bla* regulators stabilizes *mecA* expression; this is supported by the fact that >95% of MRSA carry a beta-lactamase [100]. Arede *et al*. suggest that the *mecA* expression is optimized by formation of MecI::BlaI heterodimers that have lower affinity to the *mecA* promoter resulting in derepression of the *mecA* transcription [101]. The MecI, MecR1 and MecR2 regulation system is often only partially present or

mutated in clinical strains as listed in the SCC*mec* types (Table 1). Only the SCC*mec* types II, III, VIII and XI carry an intact *mecR1-mecI* regulatory system that generally contains also the anti-repressor MecR2 [77]. There exist highly resistant strains constitutively expressing *mecA* due to the absence of the MecI repressor, as for example the strain COL [19,102].

However, the level of methicillin resistance is not only defined by *mecA* expression levels but it is also dependent on the genetic composition of a strain and on specific genetic factors influencing resistance [103-105]. There is no direct correlation between the *mecA* transcription level and the beta-lactam resistance level [104,106]. Factors influencing methicillin resistance, previously called *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) genes, are often directly or indirectly involved in cell envelope biosynthesis and turnover [107]. The majority of these factors do not directly interfere with *mecA* expression and influence often also intrinsic resistance to methicillin in MSSA strains [103,104]. Examples are the cell wall biosynthesis enzymes like FemABX [108], GlmM [109], GlmS [110], MurE [111], MurF [112], PBP2 [113], PBP4 [114] or wall teichoic acid biosynthsis enzymes including TarO/TagO [115], the *dlt* operon [116] and the predicted wall teichoic acid ligase MsrR [117]. Factors with indirect or with no obvious connection to the cell envelope include regulators like SigB [102], SpoVG [118], *agr* [119], SarA [119], XdrA [120], CcpA [121], SecDE [122] and the two-component system VrasR ([123,124], see section 1.3.2). Recently, the nucleotide second messengers c-di-AMP and (p)ppGpp were identified to influence beta-lactam resistance as well ([125,126], see section 1.5).

1. 3 Two-component systems of S. aureus

1. 3. 1 Overview of the two-component systems of *S. aureus*

For the survival of bacteria it is crucial to respond to changes in the environment. One mechanism by which *S. aureus* senses exogenous signals and respond to the stress stimuli by cellular adaption are two-comment systems (TCS). Signal transduction is facilitated by the phosphor transfer from a membrane located senor kinase to the response regulator that then generally regulates genes on the transcriptional level. Dephosphorylation of the regulator shuts down the regulatory response. The best characterised TCS of *S. aureus* is the *agr* system that globally regulates virulence and toxin expression in a growth phase-dependent and cell density dependent manner (quorum sensing) (reviewed in [127]). *S. aureus* possesses 16 chromosomally encoded TCS that are summarized in Table 2. Only the WalKR TCS is essential and at least five TCS are reported to influence antibiotic resistance: VraSR, BecRS, WalKR, LytRS and GraSR; they have effects on beta-lactam, bacitracin and/or glycopeptide resistance (Table 2, [128-130]). In addition to the chromosomally encoded TCS *S. aureus* can acquire TCS encoded on mobile genetic elements, such as the VanSR, BlaR1-BlaI and MecR1-MecI, involved in regulation of vancomycin and beta-lactam resistance, respectively (section 1.2.2.4 [46]).

Table 2: Two-component systems of *S. aureus*

Name	ORF no.	function/predicted role/connection to antibiotic resistance	References
AgrAC	MW1962/3	accessory gene regulator, regulation of virulence, quorum sensor	[127]
VraSR	MW1824/5	regulation of cell wall biosynthesis enzymes and autolysins, protection to cell wall stress including various cell wall active antibiotics	[124,131,132]
LytSR	MW0236/7	regulation of autolysins and sensing of alteration of membrane potential, influence on penicillin tolerance and resistance to cationic antimicrobial peptides	[133,134]
SaeRS	MW0667/8	regulation of virulence factors	[135]
WalKR*	MW0018/9	regulation of autolysins, virulence genes, methicillin resistance and glycopeptide intermediate resistance	[128,136]
ArlRS	MW1304/5	regulation of virulence factors and autolysin	[137]
SrrAB	MW1445/6	regulation of virulence, respiratory response, anaerobic genes	[138]
PhoPQ	MW1636/7	alkaline phosphatase synthesis response regulator	[130]
KdpDE	MW2002/3	regulation of virulence, potassium transport	[139]
HssRS	MW2282/3	regulation of response to heme (heme transporter HrtAB)	[140]
NreBC	MW2313/4	Regulation of dissimilatory reduction and transport of nitrate and nitrate, oxygen-responsive	[141]
Aps/GraSR	MW0621/2	regulation of *dlt* and *mprF,* influence on resistance to vancomycin, gentamicin, nisin and defensins	[142,143]
BceRS	MW2544/5	regulation of two transporters connected to bacitracin susceptibility	[129]
SA0215/6	MW0198/9	uncharacterised TCS	[130]
SA1158/9	MW1208/9	uncharacterised TCS	[130]
SA1666/7	MW1789/90	uncharacterised TCS	[130]

* Essential TCS

1. 3. 2 The VraSR-dependent cell wall stress stimulon

The first layer of protection from environmental stress situations is the thick cell envelope of *S. aureus.* Accordingly, adaptations to cell envelope stress in form of stress responses are critical for the survival. *S. aureus* has one cell wall stress stimulon (CWSS), a set of genes that is regulated by the VraSR two-component system [124,132,144]. The CWSS is induced by cell wall active antibiotics or by depletion of genes essential for cell wall biosynthesis like MurA, MurZ, MurB, MurF and PBP2 [131,145,146]. Although VraSR was named after its role in vancomycin resistance (vancomycin resistance associated), deletion of VraSR also decreases resistance to methicillin, fosfomycin, bacitracin and other cell wall antibiotics, indicating a crucial role of the stress response in various resistance phenotypes [147]. The core genes regulated by VraSR comprise a regulon of about 50 genes [131]. The majority of these genes encode proteins linked to cell envelope biosynthesis such as: MurZ (MurA isozyme), involved in the early steps of cell wall biosynthesis [145,148]; PBP2*,* a penicillin-binding protein (PBP) with both transglycosylation and transpeptidation activity [113,148]; SgtB, involved in transglycosylation [148,149]; FmtA, a PBP with low affinity to beta-lactams [150]; the

predicted autolysins Atl and SA0424 [151] as well as the predicted wall teichoic acid ligases MsrR, SA0908 and SA2103 ([152,153], see also section 1.5). Up-regulation of CWSS genes directly linked to cell envelope synthesis and down-regulation of autolysins is suggested to protect against cell envelope stress [154-156]. This hypothesis is supported by increased cell wall thickness of both clinical glycopeptide intermediate resistant *S. aureus* (GISA) strains carrying VraS mutations and laboratory strains artificially over-expressing VraS resulting in an increased CWSS expression [157]. Besides well characterized genes, there are several genes of unknown or poorly characterised functions in the VraSR regulon, including the highest up-regulated gene *sas016* (also called *vraX*) of unknown function [132,158]. The exact composition of the VraSR regulon is strongly dependent on the induction conditions and the experimental procedures applied.

The signalling by VraSR consists of a typical TCS mechanism as described above (see 1.3.1.). An unknown signal for cell wall stress triggers the intramembrane histidine kinase VraS to activate the response regulator VraR by phosphorylation. In the absence of a stress signal, VraR is deactivated by VraS dependent dephosphorylation. Analysis of the phosphorylation properties revealed that VraSR might respond very fast *in vivo* [159]. In depth analysis of the DNA-binding properties of VraR to the promoter of the *vra* operon identified the VraR DNA-binding motif ACTaaAGTaTGAacaTCA [159,160]. Also *in silco* analysis of promoters of the VraSR regulon suggested the VraR DNA-binding motif TxxxxCxxxxGxxxxA [161] that is partially overlapping with the above stated motif identified by DNA-binding analysis of the *vra* operon promoter [159,160].

The VraSR TCS is homologous to the cell envelope stress sensor system LiaSR (or CesRS) present in most Firmicutes including *Bacillus subtilis* [162], *Streptococcus mutans* [163], *Streptococcus pneumonia* [164], *Enterococcus faecium* [165] as well as *Lactococcus lactis* [161]. Even though all these systems respond to cell envelope stress including an overlap of inducing antibiotics, the composition and size of their respective regulons are diverse, indicating a species specific function and relevance [144]. In *B. subtilis* and *S. mutans* a third component, LiaF, was recently discovered to repress LiaSR signalling under non stress conditions. Due to the crucial role of LiaF for signalling it was suggested that the LiaSR and homologous TCS to LiaSR are in fact a three-component systems (LiaFSR) [166]. VraSR is suggested to form a three component system including *vraT* (*sa1702*, previously called *yvqF*) that is encoded directly upstream of *vraS* in the four-cistron autoregulatory VraSR operon (*vraU-vraR-vraS-vraR*). The function of the LiaF homologue VraT, however, is still debated. McCallum *et al.* suggest that VraT actives VraSR in *S. aureus*, the opposite effect of the repressor LiaF in *B. subtilis* [147]. Their study detected that VraT interacts with VraS as well as that in a *vraT* deletion mutant both the resistance to cell wall active antibiotics and the expression of the CWSS are decreased, similar to VraS and VraR mutants [147]. Contradictory, a study analysing vancomycin intermediate resistant *S. aureus* (VISA) suggested that VraT functions as repressor

similar to LiaF [157]. A point mutation in *vraT* in a VISA strain increased vancomycin resistance and CWSS expression that was complemented by introducing the wild type VraT supporting a repressor function [157]. However, there was no direct proof of a VraT repressor function and a gain of function mutation could explain the same observations. The most recent study supports a CWSS inducing role of VraT confirming the results from McCallum *et al.* [157,167]. Transcription analysis of a *vraT* mutant showed overlapping up- and down-regulation patterns to a *vraS* mutant, and oxacillin resistance and CWSS expression was reduced in the *vraT* mutant. The function of the product of the forth gene on the *vra* operon VraU (previously called *orf1*) remains elusive, but a direct connection to VraSR seems unlikely since a *vraU* mutant did neither influence oxacillin resistance nor expression of CWSS genes [147,167].

All findings together confirm a direct involvement of VraT in VraSR signal transduction and support the hypothesis of a VraTSR three-component system. Although the exact signalling pathway is not completely understood, a working model of the VraSR system including VraT is shown in Figure 5. Upon an unknown cell wall stress signal VraS phosphorylates VraR. VraT influences the signal transduction of the system by interaction with VraS and possibly transmitting the initial stress signal to VraS. Phosphorylated VraR regulates expression of VraR regulon genes and autoregulates the expression of the *vraU-vraT-vraS-vraR* operon.

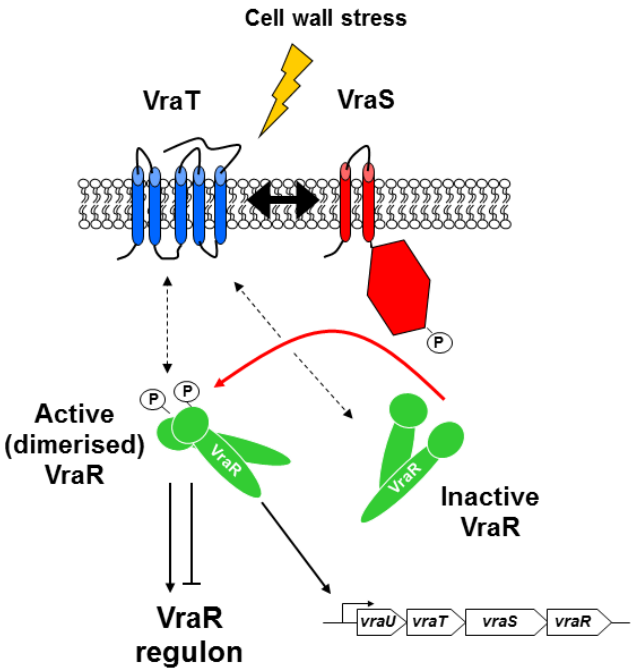

Figure 5: Working model of VraSR signalling including factor VraT. Black arrows and the black blocked arrow indicate transcriptional regulation, the red arrow indicates phosphorylation of VraR by VraS, double-pointed arrows indicate confirmed interaction of VraT and VraS; dashed double-pointed arrows indicate possible additional interactions or signal transduction pathways. Adapted from [147,167].

1. 4 Wall teichoic acid biosynthesis and the LytR-CpsA-Psr protein family

Wall teichoic acids are anioic glyopolymers that are covalently attached to the peptidoglycan by phosphodiester linkage to C6 hydroxyl of the *N*-acetyl muramic acid. Wall teichoic acids make up to 60% of the total cell wall mass of Gram-positive bacteria. In contrast to *B. subtilis* that synthesizes ploy(glycerol phosphate) WTA and poly(ribitol phosphate) WTA, *S. aureus* produces mainly poly(ribitol phosphate) WTA [168]. The function of wall teichoic acids is not yet fully understood. WTA are found to be involved in protection against harmful molecules and environmental stress; in extracellular interactions including adhesion and phage reception; in the stability and permeability of the membrane as well as in the control of cation concentration in the cell envelope; and they act as scaffold for extracellular enzymes involved in cell wall biosynthesis and turn over [4,168]. WTA are required in cell wall biosynthesis to direct autolysin to the septum and protect the other parts of the cells from autolysis [169].

The enzymes TarO and TarA initiate WTA synthesis by transferring UDP linked precursors *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl mannosamine (ManNAc) to the lipid carrier undecaprenyl-phosphate (or also called bactoprenol) (Figure 6, [170]). TarO and TarA deletion mutants are viable but have impaired adhesion properties and an impaired cell division [171,172]. Deletion of all following enzymes in the wall teichoic acid synthesis pathway is so-called synthetic lethal (Figure 5). This means that deletion mutants are unable to grow in a wild type background but are viable in a *tarO* or *tarA* mutant background [173]. A possible explanation for this phenomenon is that the accumulation of the intermediates is toxic or that it blocks the lipid carrier required also for cell wall biosynthesis [174]. After initiation, the next step of the biosynthetic pathways is addition of two glycerol-3-phosphates by TarB and TarF [175]. TarD generates CDP-glycerol, the activated precursor for these two steps by TarB and TarF [176]. The actual polymerisation step of ribitol-5-P is catalysed by TarL and it is dependent on CDP-ribitol precursor synthesis by TarJ and TarI [175,177,178]. Interestingly, there is a duplication of the gene cluster TarIJL in *S. aureus* and the two different variants of TarL were claimed to produce different sizes of poly(ribitol) chains [177]. The flipping of the lipid carrier attached precursor from the inside to the outside of the cell is facilitated by the transporter TarGH [179]. Even though wall teichoic acids are not essential, the deleterious effects of inhibition from the third step onwards make WTA biosynthesis a target for antibiotics. So far, only one antibiotic is described to specifically target wall teichoic acid biosynthesis, targocil, which inhibits the tranferase TarGH (Figure 5, [50,180]). Tunicamyin inhibits both the cell wall synthesis enzyme MarY as described above (Figure 3) and TarO, the latter even with a much higher affinity [173].

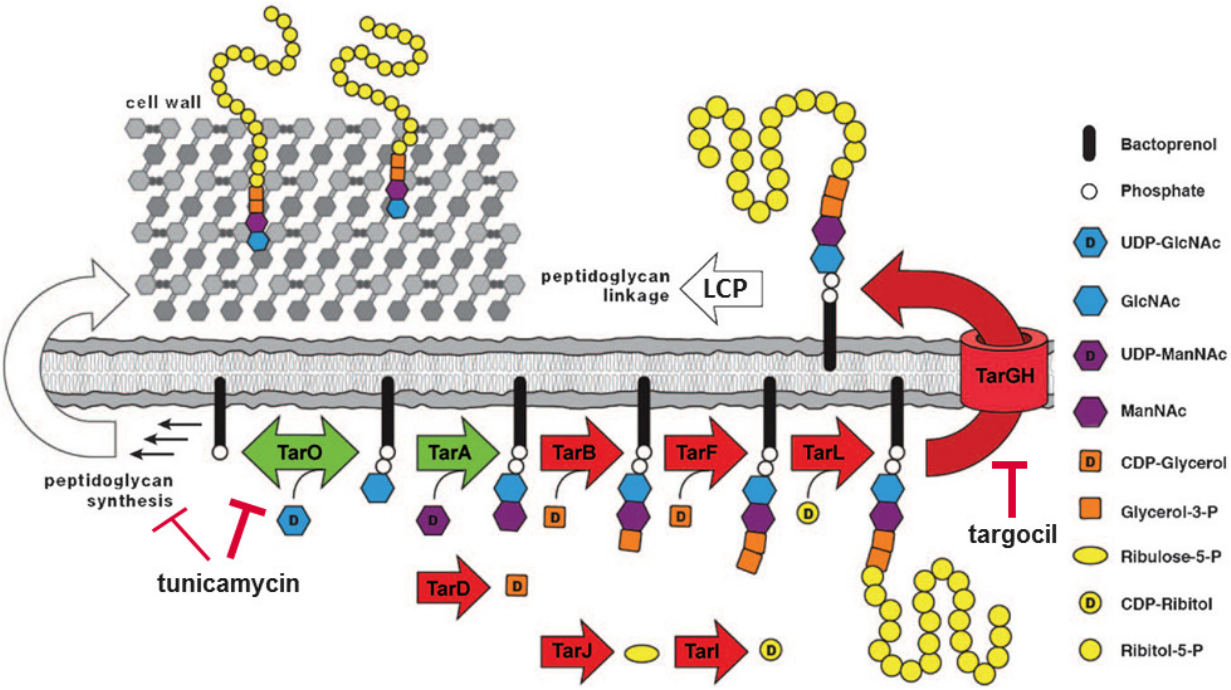

Figure 6: Schematic representation of the enzymatic steps involved in *S. aureus* wall teichoic acid (WTA) biosynthesis and antibiotics inhibiting WTA biosynthesis. Inhibition of enzymatic steps by antibiotics is indicated by red blocked arrows. Nonessential WTA synthesis enzymes are colored green, conditionally essential are colored red and the hypothetical step by LCP proteins is colored white. Adapted from [168].

The very last step of wall teichoic acid synthesis is the attachment of WTAs to the peptidoglycan. This final step was for a long time elusive and has only been described by Kawai *et al.* in *B. subtilis* in 2011 [152]. Their study revealed that members of the protein family LytR-CpsA-Psr (LCP), a protein family unique to Gram-positive bacteria [181], catalyses the ligation of wall teichoic acids to the peptidoglycan in *B. subtilis* [152]. The role of LCP proteins in other Gram-positive species is only partially described by analysis of LCP mutant stains that suggest a function connected to cell envelope synthesis [117,153,182-184]. In *S. aureus* there are three LCP proteins, SA2103, SA0908 and MrsR which are partially redundant [153]. Deletion of MsrR decreases methicillin resistance, virulence and it results in an impaired cell division [117]. Surprisingly, the LCP triple mutant in *S. aureus* is viable, in contrast to *B. subtilis* where LCP proteins are synthetic lethal [152,153]. In *B. subtilis* all three LCP genes can only be deleted in a Δ*tarO* or Δ*tarA* background that prevents the flux of precursors into the synthesis pathways, comparable to other WTA synthesis enzymes that are considered conditionally essential [152,153]. Though, the *S. aureus* LCP triple deletion is not lethal, it causes severe growth defects with irregular septum formation, defective cell separation and giant cells and leading to the speculation that the *S. aureus* LCP triple mutant is possibly only viable due to secondary mutations [153].

1. 5 Nucleotide second messengers

Nucleotides such as (p)ppGpp, cAMP, cGMP, c-di-GMP, c-di-AMP or c-di-GMP-AMP are so-called second messengers and they are involved in regulation of various cellular processes including virulence, biofilm formation, motility and the cell cycle (reviewed in [185]). Quorum sensing signalling is known to regulate similar processes and it was suggested that there is an interaction between signalling by nucleotide and quorum sensing [186]. Regulation by nucleotides take place at all stages from transcriptional and translational to post translational regulation. Nucleotide signalling is introduced here by focusing on the nucleotides (p)ppGpp and c-di-AMP that affect antibiotic resistance of *S. aureus*.

1. 5. 1 The alarmone (p)ppGpp

The two linear nucleotides guanosine tretraphosphate ppGpp and guanosine pentaphosphate pppGpp (summarized as (p)ppGpp) regulate stringent response and are termed 'alarmones' that are produced rapidly in response to amino acid, phosphate, fatty acid, carbon and iron starvation. (p)ppGpp shuts down the proliferating processes of bacteria in response to nutrient limitation for example by transcription repression of the proteins synthesis machinery including rRNA, ribosomal proteins and translation factors and by up-regulation of stress response mechanisms [187]. There are fundamental differences in the molecular mechanism of (p)ppGpp regulation between different species (reviewed in [188]). The knowledge of (p)ppGpp signalling in *S. aureus* is limited, but similar to other bacteria, a down-regulation of protein and nucleotide synthesis related genes was detected [189]. The mechanism of this down-regulation is unknown but it is suggested that the lowering of the GTP pool in stringent response through (p)ppGpp production limits transcription initiation from genes starting with iGTP as found in *B. subtilis* [190,191]. The major pathway in *S. aureus* for (p)ppGpp-dependent up-regulation of genes is the de-repression of the regulator CodY [189]. The CodY regulates mainly genes of the nitrogen metabolism but also virulence factors essentially by repression of the *agr* system [192,193]. Additionally, seven genes were detected to respond CodY-independently to (p)ppGpp levels including two phenol-soluble-modulins that are cytolytic molecules important for intracellular survival [189]. The importance of (p)ppGpp in the virulence of *S. aureus* is in agreement with similar findings in other species [194]. A recent study identified also a direct correlation of the level (p)ppGpp to methicillin resistance levels by a yet unknown mechanism [126].

1. 5. 2 The new second messenger c-di-AMP

1. 5. 2. 1 c-di-AMP synthesis and degradation

c-di-AMP is a new second messenger that was first discovered in structural analysis of the DNA integrity scanning protein DisA of *Thermotoga maritime* revealing the diadenlyate cyclase (DAC) domain five years ago [195]. c-di-AMP is synthesized by condensation of two ATP molecules by

DAC domain proteins and degraded to pApA by a specific phosphodiesterase, commonly named GdpP (Figure 7). The DAC domain is found in proteins of 275 species, predominantly in the Gram-positive phyla Firmicutes and Actinobacteria but also in Gram-negative genera Bacteroides, Cyanobacteria and Chlamydia among others, and even in certain Archaea species [196,197]. Besides DisA, there are two additional DAC domain proteins in *B. subtilis*, DacA (also called CdaA and YbbP) and DacB (also called CdaS and YojJ) (Figure 8). C-di-AMP is essential for bacteria and therefore a *B. subtilis* DisA/DacA/DacB triple mutant as well as a DisA/DacA double mutant are not viable [198]. Deletion of DacA and DisA is likely deleterious because DacB was detected to be only expressed during sporulation [198-200]. DacA is expressed in vegetative cells, contains besides the DAC domain a transmembrane domain, and it is homologous to the DAC proteins of *L. lactis*, *Listeria monocytogenes*, *S. pyogenes* and *S. aureus,* all of which possess only one protein with a DAC domain [125,201-203]. In contrast, *Mycobacterium smegmatis* possesses only one DAC domain protein homologues to DisA and a DacA protein is absent [197]. The activity of DacA in *B. subtilis* is specifically stimulated by the c-di-AMP synthase regulator CdaR (previously called YbbR), and Mehne *et al.* suggested that the same positive regulation may occur by YbbR of *S. aureus* [199]. Interesting to note is that the c-di-AMP degrading GpdP is inhibited by (p)ppGpp in *B. subtilis* connecting the signalling system of c-di-AMP with (p)ppGpp [204].

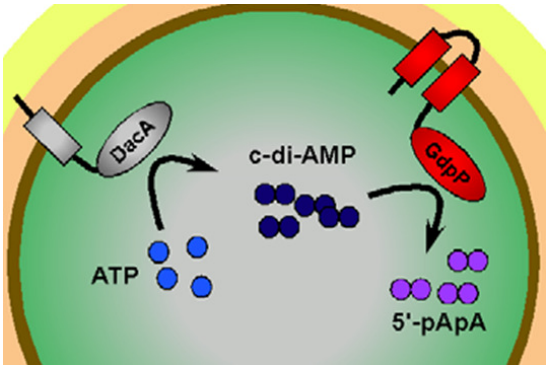

Figure 7: c-di-AMP turn over in *S. aureus.* Adapted from [125].

1. 5. 2. 2 The predicted cellular role of c-di-AMP

The cellular role and the regulatory mechanisms of c-di-AMP are not fully elucidated and only five c-di-AMP receptors have been discovered so far. In *S. aureus* a screen for c-di-AMP receptors identified the potassium transporter-gating component KtrA; the predicted cation/proton antiporter CpaA; the two-component system sensor kinase KdpD; and the hypothetical PII-like signal transduction protein PstA, as c-di-AMP binding proteins (Figure 8, [205]). KtrA and CpaA share a common domain, RCK_C (regulator of conductance of K+), found in transporters, antiporters and channels, in all kingdoms of life. KtrA interacts with a membrane component responsible for the uptake of potassium named KtrB [205]. The c-di-AMP receptor KdpD phosphorylates the response regulator KdpE that is a possible c-di-AMP signal transduction pathway [205]. DisA contains in addition to the DAC domain

a DNA-binding domain and was suggested to sense DNA damage via the reduction of cellular c-di-AMP levels in *B. subtilis* that possibly prevents entering sporulation (Figure 8, [195,206]). Targets or receptor proteins of c-di-AMP in *B. subtilis* were not identified so far. In *Mycobacterium smegmatis* a TetR-like transcription factor DarR was detected as c-di-AMP target protein and confirmed to negatively regulate three target genes and its own expression in a c-di-AMP dependent manner (Figure 8, [207]). The target genes of c-di-AMP dependent regulation by DarR are the cold shock protein CspA, a medium fatty acyl-CoA ligase and a major facilitator family transporter (Figure 8, [207]).

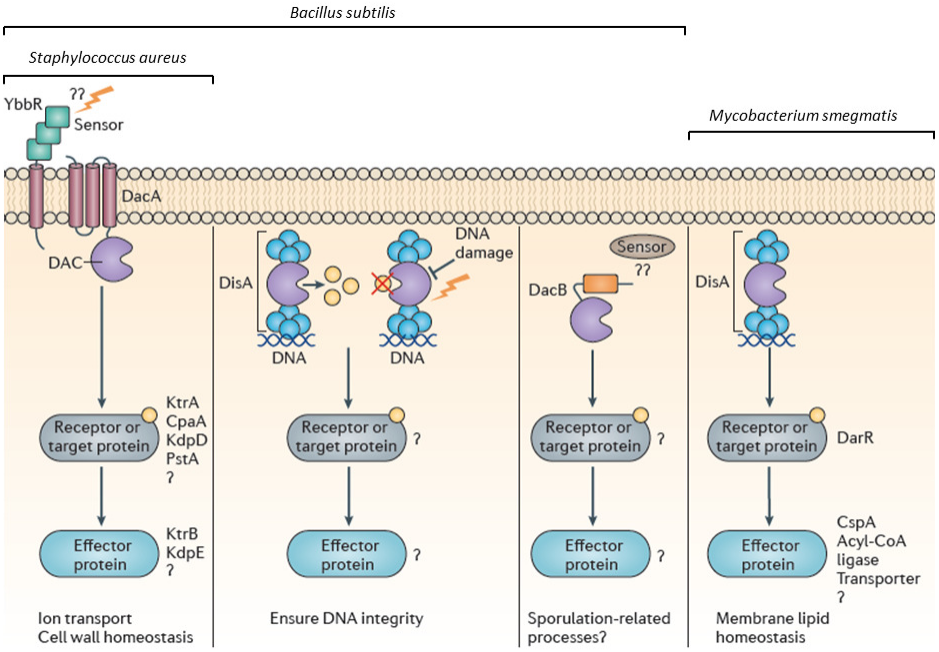

Figure 8: Overview of c-di-AMP receptor and target proteins of *S. aureus*, *B. subtilis* and *M. smegmatis*. Environmental signals are indicated as lightning bolts and c-di-AMP is shown as yellow circle. Adapted from [197].

Independently of the recently discovered receptors, various studies of *gdpP* mutants in different bacterial species showed that c-di-AMP influences cell envelope homeostasis. Point mutations in *gdpP* and deletion of *gdpP* increase beta-lactam resistance of *B. subtilis*, *L. monocytogenes, L. lactis* and both MSSA and MRSA strains [125,198,202,203,208-211]. Deletion of *gdpP* in *S. aureus* increases the c-di-AMP level up to 15-fold and results in increased peptidoglycan cross-linking, autolysis, salt sensitivity, biofilm formation and a 13% reduced cell size [125,205]. Acquisition of point mutations in *gdpP* and deletion of *gdpP* were shown to rescue the absence of the essential cell wall component lipoteichoic acids in *S. aureus*, suggesting that elevated c-di-AMP levels could be a cell envelope stress response [125]. In *L. lactis gdpP* deletion or acquisition of point mutations in *gdpP* resulte in addition to decreased penicillin sensitivity in increased temperature tolerance and decreased salt tolerance [202]. Deletion of *gdpP* (also called *pdeA*) in *L. monocytogens* increases expression of acid stress response genes [203]. Studies in *B. subtilis* and *L. monocytogenes* revealed that the c-di-AMP level is tightly regulated and that both elevated and depleted levels of c-di-AMP are detrimental for the

growth of the bacteria [199,203]. With the currently limited knowledge on the function of the identified c-di-AMP receptors, it is not possible to explain all observed phenotypes and it is likely that there might be more receptors to be discovered. However, the current knowledge from analysis of altered c-di-AMP levels suggests a cell envelope or cell envelope stress related function of c-di-AMP.

The genetic region of *dacA* of *S. aureus* and *B. subtilis* supports a connection of c-di-AMP to the cell envelope. Both *S. aureus* and *B. subtilis* form a three gene operon *dacA-ybbR-glmM* and *dacA-cdaR-glmM* [109,199], respectively, including the phosphoglucosamine mutase *glmM* that catalyses the first step of the cell wall precursor UDP-*N*-acteyl glucosamine synthesis pathway [212]. The third gene of the operon encodes the above mentioned DacA regulator CdaR and its *S. aureus* homologue YbbR, respectively [109,199]. In *B. subtilis* the glucosamine-6-phosphate synthase gene *glmS* is located directly downstream of *glmM* and an additional large transcript *dacA-ybbR-glmM-glmS* is formed [199]. In contrast, *glmS* in *S. aureus* is separated from *glmM* by about 14 kb that contains the cell wall associated protein *fmtB* (also called *mrp*) as well as mannitol synthesis and export genes [109]. A larger transcript including *fmtB* but not *glmS* has been detected in *S. aureus (dacA-ybbR-glmM-fmtB)* [213].

Besides the effects on the bacteria, c-di-AMP is a host immune stimulator and was suggested as mucosal vaccine adjuvant [214]. *L. monocytogenes* secretes c-di-AMP via two multidrug efflux pumps belonging to the major facilitator superfamily, MdrT and MdrM [215,216]. Secretion of c-di-AMP triggers a type I interferon response that is suggested to be crucial for establishing *L. monocytogenes* infections [203,215,217]. A similar immune stimulation of c-di-AMP was detected for *Chlamydia trachomatis*, the first Gram-negative bacterium confirmed to produce c-di-AMP [210]. However, whether the induction of a type I interferon response is generally an advantage for the host or the bacteria is still debated [218].

2 Aim of the study

The aim of this project was to investigate factors that influence resistance or intrinsic tolerance of *S. aureus* to antibiotics. For Project I the goal was to determine induction kinetics of the VraSR-dependent cell wall stress stimulon for various cell wall active antibiotics and to validate the role of the VraSR two-component system for intrinsic resistance to cell wall antibiotics. In Project II the role of LCP proteins in cell envelope biosynthesis and their connection to the cell wall stress stimulon and antibiotic resistance was aimed to be determined. Project III aimed to discover new factors that influence beta-lactam resistance and fitness of MRSA using a whole genome sequencing approach. The goal of Project IV was to analyse the effect of amoxicillin and minocycline administration on fitness and antibiotic resistance of the commensal *S. aureus* population in healthy volunteers.

3 Results

3.1 Project I:

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RESEARCH ARTICLE Open Access

Induction kinetics of the *Staphylococcus aureus* cell wall stress stimulon in response to different cell wall active antibiotics

Vanina Dengler, Patricia Stutzmann Meier, Ronald Heusser, Brigitte Berger-Bächi, Nadine McCallum*

Abstract

Background: *Staphylococcus aureus* activates a protective cell wall stress stimulon (CWSS) in response to the inhibition of cell wall synthesis or cell envelope damage caused by several structurally and functionally different antibiotics. CWSS induction is coordinated by the VraSR two-component system, which senses an unknown signal triggered by diverse cell wall active agents.

Results: We have constructed a highly sensitive luciferase reporter gene system, using the promoter of *sas016* (*S. aureus* N315), which detects very subtle differences in expression as well as measuring > 4 log-fold changes in CWSS activity, to compare the concentration dependence of CWSS induction kinetics of antibiotics with different cell envelope targets. We compared the effects of subinhibitory up to suprainhibitory concentrations of fosfomycin, D-cycloserine, tunicamycin, bacitracin, flavomycin, vancomycin, teicoplanin, oxacillin, lysostaphin and daptomycin. Induction kinetics were both strongly antibiotic- and concentration-dependent. Most antibiotics triggered an immediate response with induction beginning within 10 min, except for tunicamycin, D-cycloserine and fosfomycin which showed lags of up to one generation before induction commenced. Induction characteristics, such as the rate of CWSS induction once initiated and maximal induction reached, were strongly antibiotic dependent. We observed a clear correlation between the inhibitory effects of specific antibiotic concentrations on growth and corresponding increases in CWSS induction kinetics. Inactivation of VraR increased susceptibility to the antibiotics tested from 2- to 16-fold, with the exceptions of oxacillin and D-cycloserine, where no differences were detected in the methicillin susceptible *S. aureus* strain background analysed. There was no apparent correlation between the induction capacity of the various antibiotics and the relative importance of the CWSS for the corresponding resistance phenotypes.

Conclusion: CWSS induction profiles were unique for each antibiotic. Differences observed in optimal induction conditions for specific antibiotics should be determined and taken into account when designing and interpreting CWSS induction studies.

Background

Staphylococcus aureus is a major cause of both nosocomial and community-acquired infections worldwide. Because staphylococci can adapt rapidly to varying environmental conditions they are quick to develop resistance to virtually all antibiotics and multiple-drug resistance, especially in methicillin-resistant *S. aureus* (MRSA), severely restricts antibiotic therapy options. One of the major targets for antimicrobial agents is the bacterial cell envelope, which is a complex, multi-macromolecular structure that undergoes highly ordered cycles of synthesis and hydrolysis, in order to facilitate cell division while maintaining a protective barrier against environmental stresses. There are several different classes of antibiotics that target specific cell envelope structures or enzymatic steps of cell wall synthesis (Figure 1).

Many antibiotic resistance phenotypes in *S. aureus* are influenced by global regulators that control virulence factors, metabolism and/or stress responses [1]. One of the latter is the VraSR system, which triggers the cell

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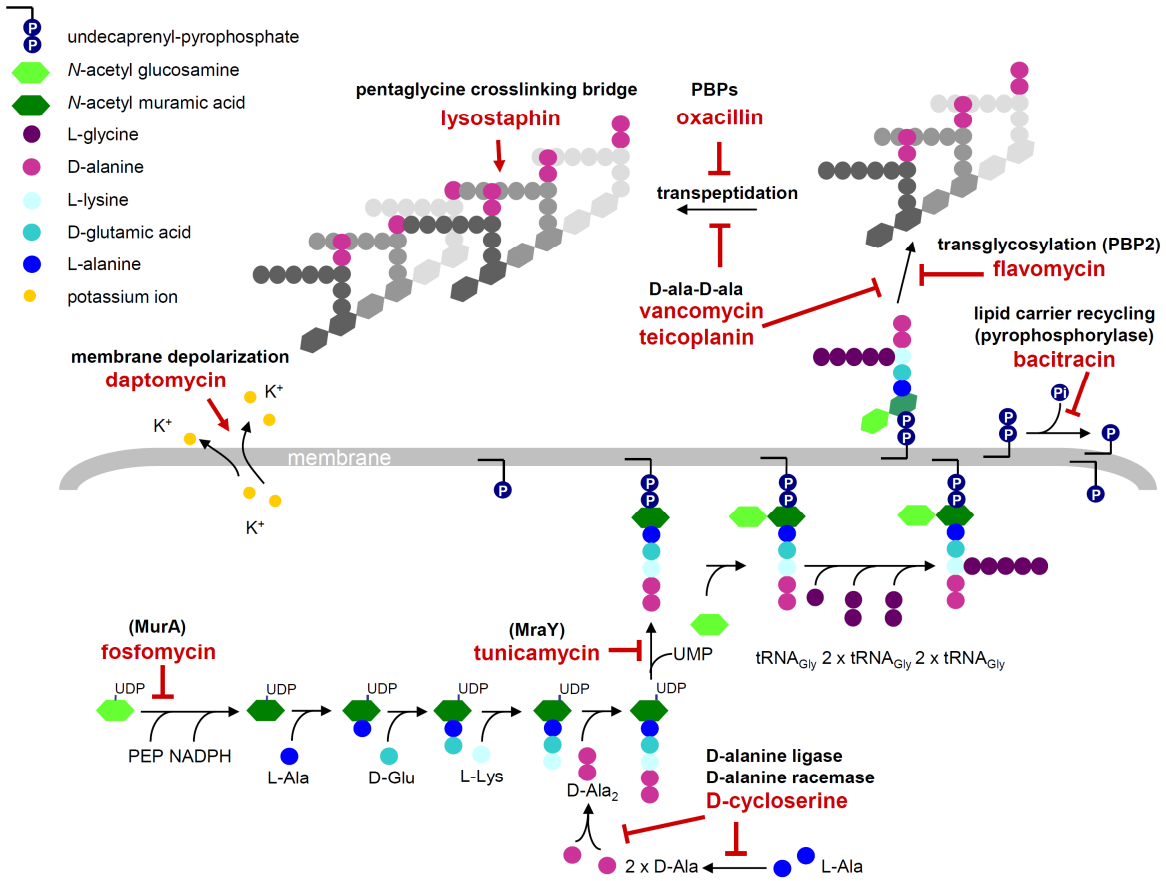

Figure 1 Schematic representation of the enzymatic steps involved in *S. aureus* cell wall synthesis and the targets of cell wall active antibiotics. Fosfomycin inhibits the enzyme MurA (UDP-*N*-acetylglucosamine-3-enolpyruvyl transferase) that catalyses the addition of phosphoenolpyruvate (PEP) to UDP-*N*-acetyl-glucosamine (GlcNAc) to form UDP-N-acetyl-muramic acid (UDP-MurNAc) [34]. D-cycloserine prevents the addition of D-alanine to the peptidoglycan precursor by inhibiting D-alanine:D-alanine ligase A and alanine racemase [35]. Tunicamycin is a glycoprotein antibiotic that inhibits the transfer of peptidoglycan precursor (phospho-MurNAc-pentapeptide) to the lipid carrier undecaprenyl pyrophosphate (or C55-isoprenyl pyrophosphate), catalysed by the translocase MraY [36,37]. Sub-lethal concentrations of tunicamycin also inhibit TarO, the first enzyme in the wall teichoic acid pathway [38,39]. Bacitracin forms a metal-dependent complex with the lipid carrier undecaprenyl pyrophosphate, thereby preventing dephosphorylation and the recycling of the lipid carrier required for cell wall synthesis [40,41]. Flavomycin (a moenomycin complex) is a phosphoglycolipid antibiotic that inhibits transglycosylation through binding of the transglycosylase domain of penicillin-binding protein 2 (PBP2) [42]. Glycopeptide antibiotics, such as vancomycin and teicoplanin, inhibit cell wall synthesis by binding the D-ala-D-ala of the lipid II and sterically hindering transglycosylation and transpeptidation. Teicoplanin activity is enhanced through its interaction with the cytoplasmic membrane [43]. ß-lactam antibiotics, such as oxacillin, bind the transpeptidase active domain of penicillin-binding proteins (PBPs) by mimicking the D-ala-D-ala end of the pentapeptide [44]. The mode of action of daptomycin is not fully known, it causes calcium-dependent disruption of membrane function and potassium efflux [45], but was also predicted to directly or indirectly inhibit peptidoglycan systhesis [9]. Lysostaphin is a zinc metalloenzyme that cleaves the pentaglycine crosslinking bridge specific for the cell wall of *S. aureus* [46]. (Adapted from [47]).

wall stress stimulon (CWSS); a set of genes that is induced in *S. aureus* upon exposure to cell wall active antibiotics, cell wall hydrolysis, or the inhibition of cell wall synthesis, but not by other external stresses, such as temperature, osmotic or pH extremes [1-3]. An unknown signal, responding to cell wall stress, stimulates the intramembrane sensor VraS to activate the response regulator VraR by phosphorylation. When the stress signal is relieved, VraR is subsequently deactivated by VraS-specific dephosphorylation [4].

VraR, depending upon its phosphorylation state, was shown to recognise VraR-responsive promoter sequences and to control the expression of target genes [5]. The phosphorylation kinetics suggested that VraSR signal transduction was likely to respond very rapidly *in vivo* [4]. A general stress signal, rather than the

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antibiotics themselves, was proposed to initiate CWSS induction [6-8]. This hypothesis is supported by the fact that the CWSS is induced by several different cell wall antibiotics with different targets and/or modes of action as well as by the inhibition of cell wall synthesis resulting from reduction of PBP2 and MurF expression [6,7,9].

Upregulation of the CWSS provides a certain level of resistance/tolerance to most VraSR-inducing agents, although the exact stress response coordinated has not been fully characterised. Core CWSS genes include: *murZ* (MurA isozyme), involved in the early steps of cell wall biosynthesis [10]; *pbp2* and *sgtB*, involved in transglycosylation; and *fmtA*, a penicillin binding protein with low affinity to β-lactams [3,11,12]. Therefore activation of the CWSS is predicted to enhance cell wall synthesis [2]. This is substantiated by the identification of clinical isolates with point mutations in the *vraSR* operon that lead to increased basal expression of the CWSS in the absence of inducing agents, with the resulting phenotypes including thickened cell walls and increased levels of glycopeptide and ß-lactam resistance [13,14].

The VraSR system of *S. aureus* has been found to be induced by a much wider range of cell wall active antibiotics than the homologous LiaRS systems of *Bacillus subtilis* and *Streptococcus mutans*, which are only induced by lipid II-interacting antibiotics and not by those that inhibit the earlier or later stages of cell wall synthesis [15-18]. However, the sizes and compositions of VraSR regulons reported so far vary quite extensively and appear to be heavily dependent upon the strains and experimental procedures used. Huge variations in levels of CWSS gene induction were found not only to be dependent upon the types of antibiotic used but also on the antibiotic concentrations [2,19,20].

In this study we created a highly sensitive reporter gene construct to indirectly measure the kinetics of VraSR-dependent signal transduction in the presence of antibiotic concentrations ranging from sub- to supra-minimum inhibitory concentrations (MIC), for a selection of antibiotics with different cell envelope targets (Figure 1). This allowed us to compare maximal induction capacities and determine optimal conditions, including concentrations and exposure times, for measuring CWSS induction by different antibiotics.

Methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria Bertani (LB) broth (Difco Laboratories), shaking at 180 rpm with a 1:5 culture to air ratio, or on LB agar plates. All optical density (OD) measurements given were taken at $OD_{600\ nm}$. Media were supplemented with the following antibiotics when appropriate: 10 μg/ml tetracycline (Sigma), 10 μg/ml chloramphenicol (Sigma), 100 μg/ml ampicillin (Sigma) or 200 ng/ml anhydrotetracycline (Vetranal). Strains were stored at -80°C in skim milk.

Susceptibility tests

The MICs of antibiotics were determined by Etest (BioMérieux) on LB plates swabbed with an inoculum of 0.5 McFarland and incubated at 37°C for 24 h. The MICs of flavomycin, D-cycloserine, tunicamycin and lysostaphin

Table 1 Strains and plasmids

Strain/ plasmid	Relevant genotype[a]	Reference/ source
S. aureus		
RN4220	Restriction-negative derivative of NCTC8325-4	[48]
BB255	NCTC8325 derivative, cured of plasmid pI524	[49]
BB255ΔVraR	BB255 containing *vraR* mutation, truncating VraR after the 2nd amino acid	This study
E. coli		
DH5α	F^- *ϕ80lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1*λ^-	Invitrogen
Plasmids		
pSP-*luc+*	Luciferase fusion plasmid, *ori* ColE1, *bla*, *luc+*; Ap^r	Promega
pBUS1	*E. coli - S. aureus* shuttle vector, *tetL* ; Tc^r	[31]
pKOR1	*E. coli - S. aureus* shuttle plasmid, for creating markerless deletions; *repF* (ts), *cat*, *attP*, *ccdB*, *ori* ColE1, *bla*, P*xyl/tetO*, *secY570*; Ap^r, Cm^r	[25]
pKOR1-VraR:: stop	pKOR1 construct containing mutant *vraR* insert with XhoI site and two inframe stop codons inserted between the 2nd and 3rd *vraR* codons.	[26]
p*sas016*$_p$-*luc+*	pBUS1 containing the *sas016* promoter-luciferase reporter gene fusion	[26]
p*tcaA*$_p$-*luc+*	pBUS1 containing the *tcaA* promoter-luciferase reporter gene fusion	This study
p*sa0908*$_p$-*luc+*	pBUS1 containing the *sa0908* promoter-luciferase reporter gene fusion	This study

[a]Abbreviations: Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

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were determined by microdilution in LB broth, essentially as recommended by the Clinical and Laboratory Standards Institute [21].

Northern Blots

Northern blots were performed as previously described [22]. Overnight cultures were diluted to OD 0.05 in pre-warmed LB containing tetracycline and grown to approximately OD 0.5. Cultures were induced with increasing concentrations of oxacillin and a control culture was grown without antibiotic treatment. Samples were taken after 20 min and 60 min of induction and total RNA was extracted as described by Cheung et al. [23]. RNA samples (7 μg) were separated in a 1.5% agarose-20 mM guanidine thiocyanate gel in 1 × TBE buffer [24]. Digoxigenin (DIG)-labelled probes were amplified using the PCR DIG Probe synthesis kit (Roche) and primer pairs SAS016.for (TCATACGTTCTATGTCTGAT) and SAS016.rev (GATCTATATCGTCTTGTAAT); and luc+ (GGCAATCAAATCATTCCGGATACTG) and luc- (ATCCAGATCCACAACCTTCGCTTC).

Construction of *vraR* mutant

The pKOR1 system developed by Bae et al. [25] was used to inactivate VraR in BB255, by inserting an XhoI site and two stop codons in-frame into the beginning of the *vraR* coding sequence, truncating VraR after the 2nd amino acid, as previously described [26].

Luciferase reporter gene fusions

Promoter regions of *sas016* (SACOL0625), *tcaA* and *sa0908* (SACOL1065) were PCR amplified from *S. aureus* strain COL using primer pairs: *sas016*.lucF (AATTAGGTACCTGGATCACGGTGCATACAAC) and *sas016*.lucR (AATTACCATGGCCTATATTACCTCCTTTGC); *tcaA*.lucF (TAATGGTACCAGTATTAGAAGTCATCAATCA) and *tcaA*.lucR (TAAT CCATGGTTTCACCTCAAT TCTGTTCCT), and *sa0908*.lucF (AATTAGGTACCA TAA TAGTACACACGCATGT) and *sa0908*.lucR (TTAATCCATGGTTGATGCTCCTA TATTAAATT), respectively. PCR products were digested with Asp718 and NcoI and ligated directly upstream of the promoterless luciferase (*luc*+) gene in vector pSP-*luc*+ (Promega). Fragments containing the resulting promoter-*luc*+ translational fusions were then excised with Asp718 and EcoR1 and cloned into the *E. coli - S. aureus* shuttle vector pBUS1 (Table 1). The fusion plasmids p$tcaA_P$-*luc*+, p$sa0908_P$-*luc*+ and p$sas016_P$-*luc*+ (Table 1) were then electroporated into *S. aureus* RN4220 before being transduced, by phage 80α, into *S. aureus* BB255.

Luciferase assays for quantification of promoter induction

For induction assays, pre-warmed LB broth was inoculated with an overnight culture to an OD of 0.05. Cultures were grown to OD 0.3 - 0.5 and pre-induction samples were collected before the cultures were induced with increasing concentrations of the antibiotics: fosfomycin (disodium salt, Sigma), D-cycloserine (Sigma), bacitracin (from *Bacillus lincheniformis*, Sigma), vancomycin (Vancocin, Eli Lilly), teicoplanin (Hoechst Marion Roussel), oxacillin (InfectoPharm), flavomycin (BC Biochemie GmbH), daptomycin (Cubist Pharmaceuticals), tunicamycin (AG Scientifics) and lysostaphin (ambicin, AMBI). Medium was supplemented with 25 μg/ml ZnCl for bacitracin, 50 μg/ml $CaCl_2$ for daptomycin and 25 μg/ml glucose-6-phosphate for fosfomycin experiments. Samples were then collected and the OD measured after 10, 20, 30, 45, 60 and 120 min. For each sample, 1 ml of culture was harvested by centrifugation and the pellets frozen at -20°C. To measure the luciferase activity, pellets were thawed briefly and resuspended in PBS (pH 7.4) to an OD of either 10 or 1, depending on induction levels. Aliquots of the cell suspensions were then mixed with equal aliquots of Luciferase Assay System substrate (Promega) and luminescence was measured for 15 s after a delay of 3 s on a Turner Designs TD-20/20 luminometer (Promega) in relative light units (RLU).

For the determination of colony forming units per millilitre (CFU/ml), 1 ml samples of cultures that had been induced for 120 min with 1xMIC of each antibiotic were harvested by centrifugation. Cell pellets were resuspended in 0.85% NaCl and immediately diluted and plated on sheep-blood agar plates.

Results and Discussion

Comparison of CWSS reporter constructs

To quantify CWSS induction and follow its time course upon antibiotic exposure, the promoters of the three representative CWSS genes *sas016*, *sa0908* and *tcaA*, were fused to the luciferase reporter gene and the resulting plasmids were introduced into antibiotic susceptible strain BB255. *sas016* encodes a hypothetical protein of unknown function and was the open reading frame (ORF) found to be most strongly up-regulated by cell wall antibiotics in several studies [3,11,20]; *tcaA* encodes a predicted membrane protein that influences glycopeptide resistance and virulence in a nematode model and belongs to the core *S. aureus* CWSS [11,22,27]; and *sa0908* encodes an envelope protein that influences lytic behaviour in *S. aureus* and is one of a family of three LytR-CpsA-Psr proteins that are all induced by cell wall stress (unpublished results). Increasing concentrations of oxacillin were added to exponentially growing cultures containing reporter plasmids, and the OD and luciferase activities were measured over a two hour period (Figure 2). The three promoters were all induced in a concentration dependent manner, with induction lag times becoming shorter

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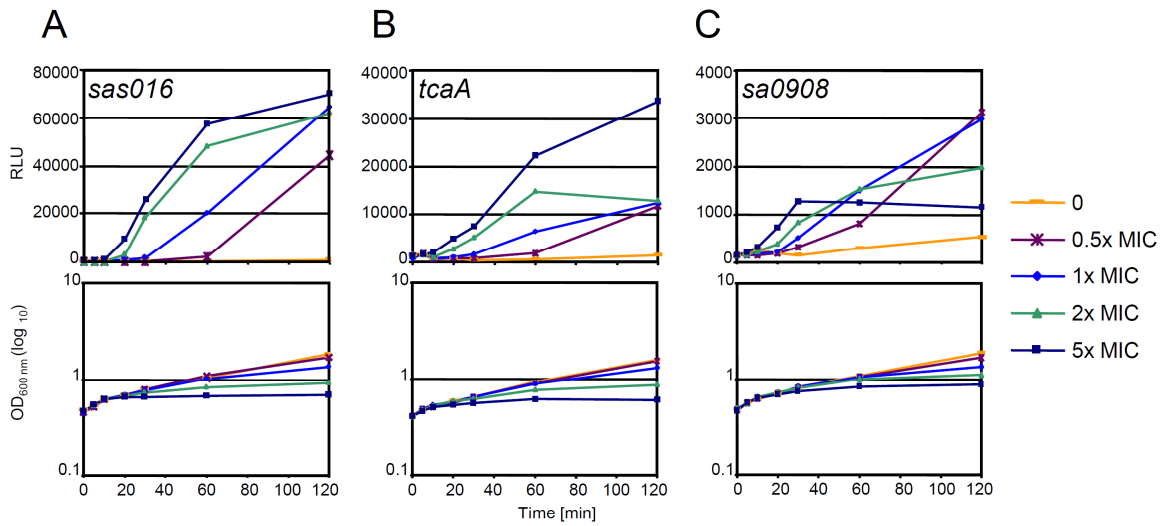

Figure 2 Induction kinetics of three CWSS promoters in response to varying concentrations of oxacillin. Luciferase activities and growth curves of strain BB255 containing: A, p*sas016-luc+;* B, p*sa0908-luc+;* and C, p*tcaA-luc+;* after addition of 0, 0.5, 1, 2 or 5-fold the MIC of oxacillin at time point zero.

and induction rates steeper as oxacillin concentrations increased. This was mirrored by a corresponding stepwise decrease in growth rates. Induction rates generally began to slow after 60 min, upon the onset of oxacillin induced lysis [28], but again this was concentration dependent with induction rates beginning to decrease earlier in cultures with higher oxacillin concentrations.

Previous findings, using Northern blots to measure oxacillin induction levels of *sas016* after 30 min, indicated that inhibitory concentrations of oxacillin were required for induction [20]. Figure 2 confirmed that the sub-inhibitory concentration of 0.5x MIC did not noticeably induce promoter activity after 30 min, however, luciferase activity from all three promoters began to increase sharply after 60 min and continued to rise up to the final sampling point of 120 min.

Although all three promoters displayed similar relative concentration- and time-dependent induction kinetics, the *sas016* promoter produced the highest levels of luciferase activity, resulting in greater fold-changes between samples and making it the most sensitive of the three reporters. Therefore we chose the *sas016* promoter-luciferase fusion construct as the best indicator to compare induction characteristics of different cell wall active antibiotics.

Correlation between *sas016* transcript induction and luciferase activity from p*sas016*$_p$-*luc+*

To confirm that levels of luciferase activity from p*sas016*$_p$-*luc+* accurately represented levels of *sas016* gene expression, Northern blots were performed on BB255 p*sas016*$_p$-*luc+* RNA samples extracted from cultures grown using the same conditions and oxacillin concentrations used for luciferase assays. Samples were harvested 20 min and 60 min after antibiotic induction and hybridized with *sas016* and *luc+* specific DIG probes (Figure 3). Northern blots showed identical patterns of transcriptional induction for both the chromosomal *sas016* gene and the luciferase gene under the control of the *sas016* promoter in p*sas016*$_p$-*luc+*. Induction of both transcripts was highly oxacillin-concentration dependent and transcript intensities increased over time becoming stronger after 60 min than after 20 min, correlating very well with concentration-specific induction curves from luciferase assays (Figure 2).

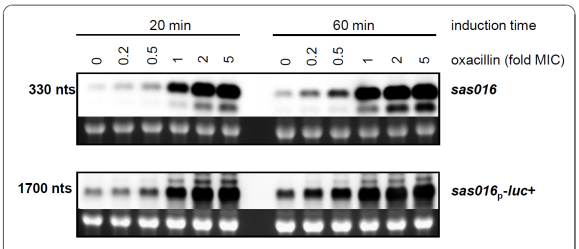

Figure 3 Northern blot analysis of *sas016* and *sas016*$_p$-*luc+* transcript induction BB255 p*sas016*$_p$-*luc+*. RNA was harvested from cultures after 20 and 60 min of induction with 0, 0.2, 0.5, 1, 2 or 5-fold MIC concentrations of oxacillin. Transcripts hybridising to *sas016* and *luc+*-specific DIG and their approximate sizes are indicated. Approximate transcript sizes are indicated on the left side of the blots. Ethidium bromide stained 16S rRNA bands are shown below Northern blots as an indication of RNA loading.

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Antibiotic-dependent induction of the CWSS

The MIC values of diverse antibiotics chosen for induction experiments were determined for strain BB255 p*sas016*$_p$-*luc*+ (Table 2). MIC concentrations were then used in induction experiments to compare the relative inducing capacities of the antibiotics (Figure 4). When adding MIC concentrations of antibiotics to exponentially growing cultures, salient differences in induction kinetics were apparent throughout the two hour sampling period, including the slopes of induction curves and the maximal luciferase activities reached. Large differences were also seen in the response of the culture's ODs over the induction period, which ranged from slight growth retardation, through to halting of growth and decreasing OD readings; reflecting differences in the effectiveness of the antibiotics and the concentrations used, which are likely to impact CWSS induction kinetics. There were no apparent connections between the stages of cell wall synthesis targeted by antibiotics and CWSS induction potential. Oxacillin and fosfomycin, which target completely different enzymatic stages of peptidoglycan synthesis, showed the highest maximal induction levels, with luciferase activity becoming induced relatively late, but then continually increasing over the two hour period. Bacitracin, tunicamycin, D-cycloserine, flavomycin and teicoplanin showed medium levels of induction, although there were large differences in the shapes of their induction curves. Bacitracin and flavomycin initiated induction very rapidly and maximal expression peaked after 60 min. The teicoplanin induction curve was shallower but maximal induction was again reached at 60 min. Vancomycin was a comparably weak inducer at the MIC concentration. Induction by lysostaphin appeared immediately, within the first 10 min, but remained very low. The OD curve for lysostaphin showed significant lysis of the culture, which would account for the overall low levels of luciferase measured. Induction therefore seems to be more strongly influenced by the specific activities of the different antibiotics used, rather than their targets.

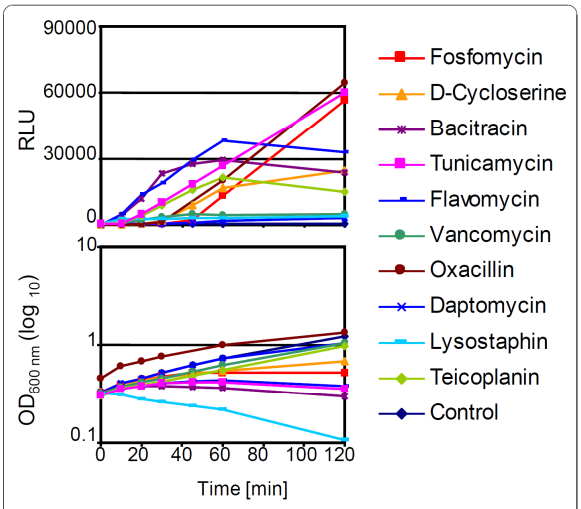

Figure 4 Antibiotic dependent induction of the cell wall stress stimulon. The upper graph shows relative light units (RLU) measured upon induction of BB255 p*sas016*$_p$-*luc*+ of cultures stressed with 1x MIC of different antibiotics. The corresponding OD values at each sampling point are presented below. The graphs shown are representative results of between two and four induction experiments performed for each antibiotic.

Concentration-dependent CWSS induction kinetics

Large differences were observed in the CWSS induction kinetics of antibiotics when used at MIC levels, however, these concentrations may not have represented the

Table 2 MIC values and summary of induction kinetics characteristics of different antibiotics

Antibiotic	MIC[a]	Fold MIC decrease in BB255ΔVraR[b]	Lag before induction[c]	Maximum induction[d]	Time point of maximum induction[e]	Concentration dependence[f]	OD/CFU/ml as % of control[g]
Fosfomycin	0.5	2x	30	high	120	high (29.5)	47/10
D-Cycloserine	12	none	10	medium	60	high (25.5)	56/36
Bacitracin	32	10x	none	medium	60	low (1.5)	26/9
Tunicamycin	8	4x	10	high	120	medium (3.0)	38/9
Flavomycin	4	16x	none	high	60	low (1.6)	41/25
Vancomycin	1.3	2x	none	low	120	medium (12.6)	100/100
Oxacillin	0.2	none	none	high	120	high (19.1)	74/20
Daptomycin	0.25	2x	none	low	120	medium (14.1)	85/75
Lysostaphin	0.065	2x	none	low	10	medium (11.3)	11/6
Teicoplanin	0.5	10x	none	medium	60	medium (7.5)	91/83

[a]Determined in μg/ml for BB255 p*sas016-luc*+. [b]Difference in MIC values of BB255/BB255ΔVraR. [c]Earliest time point at which induction was detected (min). [d]Induction levels were scored as: high (> 40'000 RLU); medium (>10'000 - < 40'000); low (< 10'000). [e]Time taken for maximum induction to be reached after antibiotic addition (min). [f]The ratio of maximal induction levels measured at 5x MIC/0.2x MIC, scored as: high (> 15); medium (>2 - < 15); low (< 2). [g] OD and CFU/ml values after treatment with antibiotics (1x MIC) for 120 min, expressed as a percentage of the values from untreated cell.

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optimal induction conditions for all of the antibiotics. Therefore, induction assays were performed as above, but using five different antibiotic concentrations ranging from sub- up to supra-inhibitory (Figure 5). Additionally, ciprofloxacin, a flouroquinolone antibiotic that does not target the cell envelope was included as a control at concentrations of 2x and 5x the MIC (MIC = 0.2 μg/ml).

Tunicamycin, flavomycin, oxacillin and fosfomycin triggered the highest maximal induction levels (RLU > 40'000) (Figure 5A, Table 2). Bacitracin, D-cycloserine, teicoplanin, and vancomycin showed medium levels of induction (RLU > 10'000 - < 40'000), while daptomycin and lysostaphin were the weakest inducers (RLU < 10'000) (Figure 5, Table 2). Daptomycin is known to

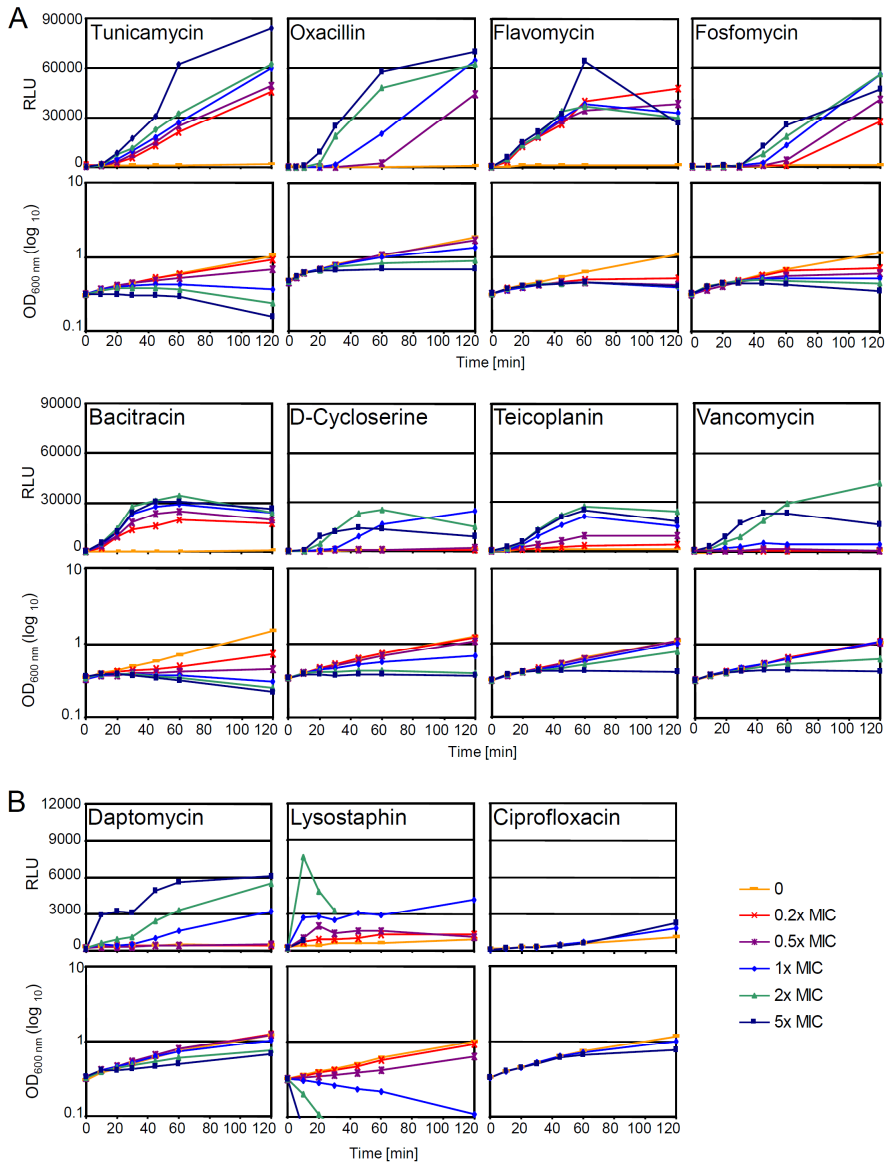

Figure 5 Concentration-dependent cell wall stress stimulon induction kinetics of different cell wall active antibiotics. Graphs show relative light units (RLU) measured upon induction of BB255 p*sas016*$_p$-*luc*+ with five different antibiotic concentrations and the corresponding OD values at each sampling point. The graphs shown are representative results of between two and four induction experiments performed for each antibiotic. A, concentration-dependent induction kinetics of antibiotics scored as high- or medium-level inducers. B, concentration-dependent induction kinetics of antibiotics scored as low-level inducers and the fluoroquinolone antibiotic ciprofloxacin.

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target the bacterial cell membrane, causing membrane depolarization and potassium efflux (Figure 1), however because of its ability to trigger the CWSS it is also thought to directly or indirectly interfere with peptidoglycan synthesis [9], although the mechanism by which this occurs is unknown.

Ciprofloxacin treated cells showed no luciferase induction after 60 min and although levels were up to 2-fold higher than in untreated cells after two hours, no further increases in expression were detected, even after four hours when the OD started to decrease in response to the ciprofloxacin treatment. Therefore marginal increases were unlikely to be caused by ciprofloxacin-specific induction of the CWSS as even the lowest inducers, lysostaphin and daptomycin, stimulated 18-fold and 14-fold induction, respectively.

Shapes of the induction curves were different for all of the antibiotics tested. Most of the antibiotics triggered immediate induction of the CWSS, with lysostaphin producing the strongest and most rapid response within the first 10 min, followed by flavomycin, bacitracin, daptomycin, vancomycin, teicoplanin and oxacillin. Contrarily, fosfomycin and D-cycloserine showed a lag phase of induction for all concentrations of approximately 30 min and 10 min, respectively, before any induction could be detected. Tunicamycin also showed a 10 min lag phase for all concentrations except 5x MIC, for which a slight 3-fold induction could be measured at the 10 min sampling point. Fosfomycin, D-cycloserine and tunicamycin act on early steps of peptidoglycan synthesis (Figure 1), which could be linked to the lags in CWSS induction. Balibar et al. also detected a lag phase of CWSS induction when *S. aureus* was treated with the UPP synthesis inhibitor hymeglusin [29].

Concentration-dependence was categorized based on the spread of the induction curves, so that antibiotics with large distances between the curves for different concentrations were scored as being highly concentration-dependent; while those in which the majority of curves clustered closely together were scored as having low dependence. The concentration-dependency of induction was also evaluated by determining the ratio of the induction measured at 5x MIC over that at 0.2x MIC (Table 2). Accordingly, fosfomycin, D-cycloserine, oxacillin, tunicamycin, vancomycin, daptomycin and lysostaphin showed relatively high concentration-dependency (ratio >2). Some of these antibiotics such as fosfomycin, oxacillin and daptomycin had quite evenly spread curves that generally increased incrementally as concentrations became higher. Whereas for vancomycin, there was a gap between the supra-MIC curves which both showed relatively high induction, and all of the sub-MIC curves that exhibited very little induction. In different experiments curves corresponding to the vancomycin MIC vacillated between showing either mid-level induction or clustering with the sub-MIC curves, indicating that the MIC of vancomycin was very close to the threshold concentration required for CWSS induction. Flavomycin and bacitracin induction curves also increased incrementally as concentrations increased, but the gaps between the curves were much smaller than for most of the other antibiotics (ratio < 2).

Previous studies have reported contradictory results regarding the induction of the CWSS by lysostaphin. Some studies detected no induction of the CWSS by lysostaphin [19,30], while Rossi et al. detected a slight induction of the CWSS gene *mrsR* upon lysostaphin treatment [31]. Possible reasons for these discrepancies are likely to be linked to experimental variations in the strains, lysostaphin concentrations and induction times used, or the sensitivity of induction detection methods. In this study, lysostaphin induction could only be detected under very specific experimental conditions (Figure 5B).

The influences of antibiotic concentrations on CWSS induction kinetics generally correlated closely with the impacts of the corresponding concentrations on the OD of the cultures (Figure 5). For example, the incremental increases in oxacillin induction curves closely mirrored corresponding decreases in culture OD curves. For flavomycin, all of the concentrations used induced luciferase activity to similar levels and all growth curves were correspondingly inhibited to similar extents. All experiments showed a definite correlation, albeit to different extents, between levels of growth arrest in the cultures and corresponding levels of CWSS induction. This trend is not always proportional, however, as bacitracin and tunicamycin OD curves showed a large degree of spread whereas induction curves were more closely clustered.

To compare how decreases in OD correlated with cell viability, CFU/ml were measured after treatment with 1x MIC of each antibiotic for two hours. The percentage decrease in CFU/ml generally corresponded well with the percentage decrease in OD (Table 2).

Impact of VraR inactivation on resistance to the cell wall antibiotics tested

Deletion of the *vraSR* operon is known to decrease resistance levels to most of its inducing antibiotics [2,6,9,32]. However, the reported effects on different resistance phenotypes varied greatly, with some MICs unaffected while others were decreased up to 40-fold; indicating that induction of the CWSS is more essential for protecting *S. aureus* against some antibiotics than others [2,6,32].

To determine if there was a link between levels or kinetics of CWSS induction and the importance of the CWSS for corresponding resistance phenotypes, we determined the MICs of BB255 compared to BB255ΔVraR for all of the antibiotics tested above and

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calculated the fold reduction in MIC (Table 2). BB255ΔVraR contains a non-polar deletion truncating VraR after the 2nd amino acid, while leaving the autoregulatory operon intact. The impact of VraR inactivation on resistance phenotypes was very similar to those previously published for deletion of *vraSR* in *S. aureus* N315 [2]. The majority of MICs decreased in the VraR mutant compared to the parent strain BB255 (Table 2). The largest impact seen was on the flavomycin MIC, which decreased 16-fold. Bacitracin and teicoplanin MICs were also much lower, with both reduced by 10-fold, and were similar to values previously published for *vraSR* null-mutants [2]. In contrast to Pietiänen et al. [32], who saw no effects on the vancomycin MIC in a *vraSR* deletion mutant of strain Newman, we observed a 2-fold decrease in vancomycin MIC, similar to that observed by Kuroda et al. in strain N315 [2]. Our results, which showed a weak 2-fold reduction in fosfomycin MIC and no impact on D-cycloserine resistance, also agreed with those obtained for the N315 *vraSR* deletion mutant. While previous reports gave conflicting results concerning the effect of VraSR inactivation on daptomycin resistance [9,32], we observed a reproducible 2-fold reduction in MIC upon VraR inactivation, supporting results from Muthaiyan et al. [9].

Inactivation of VraR had no effect on oxacillin resistance in the methicillin susceptible *S. aureus* (MSSA) strain BB255. However, inactivation of *vraR* in BB270, an MRSA isogenic to BB255 that contains a type I *SCCmec*, reduced the oxacillin MIC from >256 to 64 μg/ml [26], to similar levels as those reported for other *vraSR* mutants in MRSA strains [2,6,33]. Loss of VraR also rendered the mutant 2-fold more susceptible to the action of lysostaphin and 4-fold more susceptible to tunicamycin; phenotypes which have not been previously published for VraSR mutants.

These results confirmed that the ability to induce the cell wall stress stimulon confers varying levels of protection against the effects of cell wall active agents. However, comparison of our MIC results with our induction data revealed no clear links between how quickly, or to which maximal level, the antibiotics are able to induce the CWSS and the impact of a functional VraSR signal transduction response on resistance levels to those antibiotics.

The *sas016* promoter-luciferase fusion construct was also analysed in BB255ΔVraR. Expression levels of p$sas016_p$-*luc*+ in BB255ΔVraR in uninduced samples were ~10-fold lower than in the wild type BB255. BB255ΔVraR p$sas016_p$-*luc*+ was induced with 5x MIC of fosfomycin, D-cycloserine, tunicamycin, bacitracin, flavomycin, vancomycin, teicoplanin, oxacillin and daptomycin and 1x MIC of lysostaphin, for 60 min. The luciferase activities ranged from 1.5-fold higher to 10-fold lower than those in uninduced cultures, showing that none of the antibiotics used could induce *sas016* expression in absence of VraR.

Conclusions

In this study, we describe the application of a highly sensitive luciferase-reporter gene construct for indirectly measuring CWSS induction kinetics in *S. aureus*. This system was used to compare induction characteristics of ten different cell wall active antibiotics with diverse enzymatic targets or modes of action. Induction of the CWSS by all ten antibiotics could be precisely quantified and while all ten antibiotics induced the CWSS, induction patterns varied greatly and were highly antibiotic-specific. Each antibiotic produced unique induction curves, which differed in lag times before induction, maximal rates of induction and peak induction levels.

Induction kinetics were also strongly antibiotic concentration-dependent, to different extents for each antibiotic, and generally correlated inversely with decreasing OD values, therefore linking induction kinetics to antibiotic activity. However, there were no obvious trends linking antibiotics acting on similar stages of CWSS with specific induction patterns. Therefore, the signal triggered by all of the antibiotics, that is responsible for activating VraS signal transduction, does not appear to be linked to any particular enzymatic target, as CWSS induction was triggered equally strongly by antibiotics targeting early cytoplasmic stages (e.g. fosfomycin) and late extracellular polymerization stages (e.g. oxacillin) of peptidoglycan synthesis. This is a key difference between the VraSR system of *S. aureus* and the homologous LiaRS systems of other Gram-positive bacteria such as *B. subtilis* and *S. mutans*, which are only activated by lipid-II interacting antibiotics, such as bacitracin, ramoplanin and nisin [15-18]. The increased induction spectrum could account for the larger size of the *S. aureus* CWSS and its protective role against more different classes of antibiotics. Although no direct links between induction properties and the impact of the CWSS on respective resistance phenotypes could be found.

Previous studies have reported large differences in CWSS induction characteristics. However, most studies were performed on different strains and using different experimental conditions. Variations in characteristics observed for the ten antibiotics tested here, indicated that each antibiotic has optimal induction conditions that should be determined before CWSS studies are carried out, including the right antibiotic concentration for the strain used and the optimal sampling time point to measure maximal induction.

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Authors' contributions

VD carried out most of the experimental work and drafted the manuscript. PS and BB participated in the design and coordination of the study and helped to draft the manuscript. RH participated in the microbiological studies and helped to draft the manuscript. NM participated in the design and coordination of the study, carried out molecular biological studies and helped to draft the manuscript. All authors read and approved the final manuscript.

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3.2 Project II:

FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

Deletion of hypothetical wall teichoic acid ligases in *Staphylococcus aureus* activates the cell wall stress response

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cell wall stress stimulon; LytR-CpsA-Psr; VraSR; wall teichoic acids; ligase; *Staphylococcus aureus*.

Abstract

The *Staphylococcus aureus* cell wall stress stimulon (CWSS) is activated by cell envelope-targeting antibiotics or depletion of essential cell wall biosynthesis enzymes. The functionally uncharacterized *S. aureus* LytR-CpsA-Psr (LCP) proteins, MsrR, SA0908 and SA2103, all belong to the CWSS. Although not essential, deletion of all three LCP proteins severely impairs cell division. We show here that VraSR-dependent CWSS expression was up to 250-fold higher in single, double and triple LCP mutants than in wild type *S. aureus* in the absence of external stress. The LCP triple mutant was virtually depleted of wall teichoic acids (WTA), which could be restored to different degrees by any of the single LCP proteins. Subinhibitory concentrations of tunicamycin, which inhibits the first WTA synthesis enzyme TarO (TagO), could partially complement the severe growth defect of the LCP triple mutant. Both of the latter findings support a role for *S. aureus* LCP proteins in late WTA synthesis, as in *Bacillus subtilis* where LCP proteins were recently proposed to transfer WTA from lipid carriers to the cell wall peptidoglycan. Intrinsic activation of the CWSS upon LCP deletion and the fact that LCP proteins were essential for WTA-loading of the cell wall, highlight their important role(s) in *S. aureus* cell envelope biogenesis.

Introduction

Staphylococcus aureus mounts a general cell wall stress response in the presence of cell wall damaging agents, involving the upregulation of up to 50 genes collectively known as the cell wall stress stimulon (CWSS; Kuroda *et al.*, 2003; Utaida *et al.*, 2003; Jordan *et al.*, 2008). Induction of CWSS genes is controlled by the VraSR two-component system (Belcheva & Golemi-Kotra, 2008), which is homologous to the cell wall stress-responsive sensor-transducer systems LiaFSR of *Bacillus subtilis* (Mascher *et al.*, 2004), LiaFSR of *Streptococcus mutans* (Suntharalingam *et al.*, 2009) and CesRS of *Lactococcus lactis* (Martinez *et al.*, 2007). The sensor kinase VraS senses an unknown signal triggered by cell envelope disturbance and phosphorylates VraR, which then binds as a dimer to promoter-specific elements and facilitates transcript induction (Martinez *et al.*, 2007; Belcheva & Golemi-Kotra, 2008; Eldholm *et al.*, 2010; Belcheva *et al.*, 2012). There is a wide variation in the fold-induction levels of different CWSS genes, which is probably linked to the specificity of VraR-binding, although the exact VraR-binding consensus and the influence of specific nucleotide differences on expression and induction of different CWSS genes has not been thoroughly analysed (Martinez *et al.*, 2007; Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012).

The magnitude of CWSS induction strongly depends on the class and concentration of cell wall antibiotics (Dengler *et al.*, 2011). Disruption of wall teichoic acid (WTA) synthesis by targocil, which inhibits the WTA transporter TarG (TagG), was also shown to activate the CWSS (Campbell *et al.*, 2012). WTA are anionic glycopolymers that are attached to the peptidoglycan of

Gram-positive bacteria via a phosphodiester linkage, and they can constitute up to 60% of the total cell wall biomass. WTA of *B. subtilis* are composed of poly(glycerol phosphate) and poly(ribitol phosphate), whereas *S. aureus* contains mainly poly(ribitol phosphate) WTA. The biosynthesis of WTA is catalysed by *tag* (teichoic acid glycerol) or *tar* (teichoic acid ribitol) genes in *B. subtilis* and *S. aureus*, respectively (reviewed in Swoboda *et al.*, 2010). Besides the induction by cell wall active antibiotics, VraSR signal transduction is also triggered by internal disruption of cell wall synthesis caused by the depletion of essential cell wall biosynthesis enzymes such as MurA, MurZ, MurB (Blake *et al.*, 2009), MurF (Sobral *et al.*, 2007), PBP2 (Gardete *et al.*, 2006) or depletion of enzymes involved in mevalonate biosynthesis, the direct precursor for undecaprenyl phosphate lipid carrier synthesis (Balibar *et al.*, 2009). Induction of the CWSS enhances intrinsic resistance/tolerance to almost all cell wall damaging agents, regardless of their target or mode of action (Dengler *et al.*, 2011; McCallum *et al.*, 2011). Members of the CWSS directly linked to peptidoglycan synthesis, such as PBP2, FmtA, MurZ and SgtB, are thought to contribute to the stress response by stimulating cell wall synthesis (Cui *et al.*, 2009; Kato *et al.*, 2010; Mehta *et al.*, 2012). It is predicted that CWSS genes with unknown or poorly characterized functions are also likely to contribute to the stress response by directly or indirectly influencing cell wall synthesis.

All three *S. aureus* LytR-CpsA-Psr (LCP) genes, *msrR*, *sa0908* and *sa2103*, belong to the CWSS (Utaida *et al.*, 2003; McAleese *et al.*, 2006; Over *et al.*, 2011). LCP proteins are unique to bacteria with Gram-positive cell walls (Hübscher *et al.*, 2008; Kawai *et al.*, 2011) and typically contain a short intracellular N-terminal region, a transmembrane domain and a large extracelluar region containing the LCP domain (Hübscher *et al.*, 2008; Kawai *et al.*, 2011). Deletion of LCP proteins in *S. aureus* alters cell surface properties and decreases virulence. Phenotypes of LCP deletion mutants include defective cell separation, increased TritonX-100-induced autolysis, increased beta-lactam susceptibility, and the cell wall WTA content was reduced in an *msrR* deletion mutant (Hübscher *et al.*, 2009). Phenotypes become more pronounced in double mutants, and growth is severely impaired in the LCP triple mutant, which contains large amorphous cells with multiple septa (Over *et al.*, 2011).

Recently, the LCP proteins of *B. subtilis*, TagT (YwtF), TagU (LytR) and TagV (YvhJ) were found to be essential for the formation of a WTA-loaded cell wall. Kawai *et al.* (2011) claim that LCP proteins catalyse the final, previously uncharacterised, step in WTA synthesis, the linkage of WTA to peptidoglycan. WTA are not essential for the cell, but deletion of the first two synthesis steps, catalysed by TarA (TagA) or TarO (TagO), leads to impaired cell division, colonization and infection *in vivo* (Weidenmaier *et al.*, 2004; Weidenmaier & Peschel, 2008; D'Elia *et al.*, 2009). However, the late-acting enzymes from TarB (TagB) onwards are conditionally essential; mutants are only viable when one of the first two steps of WTA synthesis is inhibited (Swoboda *et al.*, 2010). Blocking the flux of WTA precursors into the WTA pathway prevents the deleterious sequestration of the universal undecaprenyl phosphate lipid carrier that is also essential for peptidoglycan synthesis, and it prevents the accumulation of potentially toxic intermediates. LCP proteins in *B. subtilis* are also conditionally essential, and the LCP triple mutant is only viable when *tagO* (*tarO*) is deleted (Kawai *et al.*, 2011). Whether LCP proteins fulfil the same function in *S. aureus* has not yet been verified.

In this study, reporter gene fusions were used to analyse CWSS expression levels in LCP mutants and to identify promoter regions essential for CWSS induction of LCP genes. The effect of LCP deletion on the WTA content was determined and partial complementation of the LCP triple mutant by TarO (TagO) inhibition demonstrated, suggesting that LCP proteins play an important role in the WTA decoration of *S. aureus* peptidoglycan.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C in Luria Bertani (LB) broth (Difco Laboratories), shaking at 180 r.p.m. with a 1 : 5 culture to air ratio or on LB agar plates. Optical density (OD) measurements were taken at 600 nm. Media were supplemented with the following antibiotics when appropriate: 10 μg mL^{-1} tetracycline (Sigma), 10 μg mL^{-1} chloramphenicol (Sigma), 100 μg mL^{-1} ampicillin (Sigma) or 200 ng mL^{-1} anhydrotetracycline (Vetranal).

Construction of Δ*VraR* mutants

The pKOR1 system developed by Bae & Schneewind (2006) was used to inactivate VraR in the different LCP mutant strains, by inserting an XhoI site and two stop codons in-frame into the beginning of the *vraR* coding sequence, truncating VraR after the 2nd amino acid, as previously described (McCallum *et al.*, 2011).

Northern blots

Northern blots were performed as previously described (McCallum *et al.*, 2007). To compare relative expression

Table 1. Strains, plasmids and primers

Strain/plasmid/primer name	Relevant genotype and/or phenotype (strain name) or primer sequence	Source or reference
S. aureus		
RN4220	Restriction-deficient derivative of NCTC 8325-4	Kreiswirth *et al.* (1983)
MSSA1112	Clinical isolate, *bla*, Mc^s Pen^r	Hübscher *et al.* (2009)
ΔmsrR	MSSA1112, *ΔmsrR::ermB*; Mc^s Em^r (JH100)	Hübscher *et al.* (2009)
Δsa0908	MSSA1112, marker-less *sa0908* deletion mutant (RH53)	Over *et al.* (2011)
Δsa2103	MSSA1112, marker-less *sa2103* deletion mutant (PS47)	Over *et al.* (2011)
Δsa0908/msrR	MSSA1112, *Δsa0908/msrR* double-mutant (RH72)	Over *et al.* (2011)
Δsa2103/msrR	MSSA1112, *Δsa2103/msrR* double-mutant (PS60)	Over *et al.* (2011)
Δsa2103/sa0908	MSSA1112, *Δsa2103/sa0908* double-mutant (PS109)	Over *et al.* (2011)
Δsa2103/sa0908/msrR	MSSA1112, *Δsa2103/sa0908/msrR* triple-mutant (PS111)	Over *et al.* (2011)
ΔVraR	MSSA1112, truncated VraR after the 2nd amino acid (=*ΔVraR*) (PS199)	This study
ΔVraR/msrR	MSSA1112, *ΔVraR/msrR* double-mutant (RH194)	This study
ΔVraR/sa0908	MSSA1112, *ΔVraR/sa0908* double-mutant (PS202)	This study
ΔVraR/sa2103	MSSA1112, *ΔVraR/sa2103* double-mutant (RH191)	This study
ΔVraR/sa0908/msrR	MSSA1112, *ΔVraR/sa0908/msrR* triple-mutant (NM776)	This study
ΔVraR/sa2103/msrR	MSSA1112, *ΔVraR/sa2103/msrR* triple-mutant (RH193)	This study
ΔVraR/sa2103/sa0908	MSSA1112, *ΔVraR/sa2103/sa0908* triple-mutant (RH216)	This study
SA113	Restriction-deficient derivative of NCTC 8325 (ATCC 35556)	Iordanescu & Surdeanu (1976)
SA113ΔtarO	SA113, *ΔtarO::ermB*; Em^r	Weidenmaier *et al.* (2004)
E. coli		
DH5α	F^- φ80d/acZΔM15 *recA1*	Invitrogen
Plasmids		
pKOR1	*S. aureus-E. coli* shuttle vector, *ori* pAMα1, *ori* ColE1, *E. coli* Am^r, *S. aureus* Cm^r	Bae & Schneewind (2006)
pKOR1-VraR::stop	pKOR1 construct containing mutant *vraR* insert with XhoI site and two inframe stop codons inserted between the 2nd and 3rd *vraR* codons.	McCallum *et al.* (2011)
pGC2	*E. coli–S. aureus* shuttle plasmid, *ori* ColE1-*ori* pC194 *bla cat;* *E. coli* Am^r, *S. aureus* Cm^r	Skinner *et al.* (1988)
p*msrR*	pGC2 containing 1.3-kb fragment comprising the *msrR* ORF and upstream flanking sequence	Hübscher *et al.* (2009)
p*sa0908*	pGC2 containing 1.9-kb fragment comprising the *sa0908* ORF and upstream flanking sequence	Over *et al.* (2011)
p*sa2103*	pGC2 containing 2.1-kb fragment comprising the *sa2103* ORF and upstream flanking sequence	(Over *et al.* (2011)
pBUS1	*S. aureus – E. coli* shuttle vector, *tetL;* Tc^r	Rossi *et al.* (2003)
p*sas016*$_p$-*luc*+	pBUS1 containing the *sas016* promoter-luciferase reporter gene fusion	McCallum *et al.* (2011)
p*vra*$_p$-*luc*+	pBUS1 containing the *vraSR* operon promoter-luciferase reporter gene fusion	This study
p*msrR*$_p$-*luc*+	pBUS1 containing the *msrR* promoter-luciferase reporter gene fusion	Over *et al.* (2011)
p*sa0908*$_p$-*luc*+	pBUS1 containing the *sa0908* promoter-luciferase reporter gene fusion	Dengler *et al.* (2011)
p*sa2103*$_p$-*luc*+	pBUS1 containing the *sa2103* promoter-luciferase reporter gene fusion	Over *et al.* (2011)
p*sas016Δ6bp*$_p$-*luc*+	pBUS1 containing the *sas016* promoter with 6-bp deletion fused to the luciferase gene (Fig. 2)	This study
p*sas016Δ6Bbp*$_p$-*luc*+	pBUS1 containing the *sas016* promoter with 6-bp deletion variant B fused to the luciferase gene (Fig. 2)	This study
p*msrRΔ12bp*$_p$-*luc*+	pBUS1 containing the *msrR* promoter with 12-bp deletion fused to the luciferase gene (Fig. 2)	This study
p*msrRΔ18bp*$_p$-*luc*+	pBUS1 containing the *msrR* promoter with 18-bp deletion fused to the luciferase gene (Fig. 2)	This study
p*sa0908Δ6bp*$_p$-*luc*+	pBUS1 containing the *sa0908* promoter with 6-bp deletion fused to the luciferase gene (Fig. 2)	This study
p*sa2103Δ6bp*$_p$-*luc*+	pBUS1 containing the *sa2103* promoter with 6-bp deletion fused to the luciferase gene (Fig. 2)	This study
Primers		
vra.lucF	AATTTGGTACCGCACATGTACTTAATTACTT	This study
vra.lucR	ATTAACCATGGCTATCACCTTTTATAATAAGT	This study

Table 1. Continued

Strain/plasmid/primer name	Relevant genotype and/or phenotype (strain name) or primer sequence	Source or reference
sas016.lucF	AATTAGGTACCTGGATCACGGTGCATACAAC	McCallum *et al.* (2011)
sas016.lucR	AATTACCATGGCCTATATTACCTCCTTTGCT	McCallum *et al.* (2011)
sas016-Δ6bp.F	AAATTAAGCTTGTTGATGTCACACATAAAAAT	This study
sas016-Δ6bp.R	AAATTAAGCTTTATCAACTTTTTATCAGAC AT	This study
sas016-Δ6bpB.F	AAATTAAGCTTTTCTATGTCTGATAAAAAGTT	This study
sas016-Δ6bpB.R	AAATTAAGCTTATTTACTAAGACTATTTATGT	This study
JR13 (msrR.lucF)	GGGTACCTGAGCTAAAGTTAAGTCGCC	Rossi *et al.* (2003)
JR14 (msrR.lucR)	TATCCATGGTTACCTACCTTATATCTTC	Rossi *et al.* (2003)
msrR-Δ12bp.F	AATTTAAGCTTTTATTAAGAAATCACTTGCTT	This study
msrR-Δ18bp.F	AATTTAAGCTTAGAAATCACTTGCTTTTTGAA	This study
msrR-Δ12bp/Δ18bp.R	AATTAAAGCTTTCTAATGAAAGGATGTCAAA	This study
sa0908.lucF	AATTAGGTACCATAATAGTACACACGCATGT	Dengler *et al.* (2011)
sa0908.lucR	TTAATCCATGGTTGATGCTCCTATATTAAATT	Dengler *et al.* (2011)
sa0908-Δ6bp.R	AATTTAAGCTTTTCCTTGTAATTTGAATGTTT	This study
sa0908-Δ6bp.F	AATTTAAGCTTCATAACATTTGTATTTTTTAC	This study
lucF.sa2103	GGGGTACCAAAATGACGACTTTAGATGGTAAG	Over *et al.* (2011)
lucR.sa2103	CATGCCATGGCAATCCCACCACTCCTTTACTATTCC	Over *et al.* (2011)
sa2103-Δ6bp.F	AATTAGAATTCAAGTATAGTAAAAAAATTAT	This study
sa2103-Δ6bp.R	AATTAGAATTCACGTATAACTATTTTTTATC	This study
SAS016.PErev	CTTCATGGTGATACTGTCGATA	This study

Am, ampicillin; Cm, chloramphenicol; Em, erythromycin; Mc, methicillin; Pen, penicillin; Tc, tetracycline; r, resistant; s, susceptible. Restriction sites are underlined.

levels of *sas016* in wild type and mutant strains, overnight cultures were diluted to OD 0.05 in prewarmed LB broth and cultures grown to OD 1.5, except for the LCP triple mutant that was sampled at OD 0.5 because of its severe growth defect. Uninduced culture samples were collected, and the remainder of the culture was induced with oxacillin (10 μg mL^{-1}) for 30 min before induced samples were collected. Total RNA was extracted as described by Cheung *et al.* (1994). RNA samples (9 μg) were separated in a 1.5% agarose-20 mM guanidine thiocyanate gel in 1× TBE buffer (Goda & Minton, 1995). The *sas016* digoxigenin (DIG)-labelled probe was amplified using the PCR DIG Probe synthesis kit (Roche) as previously described (Dengler *et al.*, 2011).

Primer extension

The transcriptional start site of *sas016* was determined by primer extension, as previously described (McCallum *et al.*, 2007), using primer SAS016.PErev (Table 1) and 20 μg of RNA harvested from a culture of *S. aureus* COL that had been grown to OD 0.5 and induced with 10 μg mL^{-1} of teicoplanin for 30 min.

Luciferase reporter gene fusions

The promoter region of the *vraSR* operon was PCR amplified from *S. aureus* strain COL using primer pair vra.lucF and vra.lucR (Table 1). The PCR product was digested with Asp718 and NcoI and ligated directly upstream of the promoterless luciferase (*luc*+) gene in the vector pSP-*luc*+ (Promega). Fragments containing the resulting promoter-*luc*+ translational fusions were then excised with Asp718 and EcoRI and cloned into the *Escherichia coli* – *S. aureus* shuttle vector pBUS1 (Table 1). The fusion plasmids pvra_p-*luc*+ and p$sas016_p$-*luc*+ (McCallum *et al.*, 2011) were then electroporated into *S. aureus* RN4220, re-isolated and electroporated into *S. aureus* SA113, SA113*ΔtarO*, MSSA1112 and all LCP and VraR/LCP mutants.

Predicted VraR-binding sites of luciferase fusion constructs were disrupted by amplifying each promoter as two fragments, using primers listed in Table 1. Complementary fragments were digested and ligated together, to create recombinant promoters in which 6–18-bp regions were replaced by restriction sites. Promoters were then fused to the luciferase gene as described above, and the resulting plasmids were electroporated into RN4220.

Luciferase assays

To measure luciferase activities, cultures were grown from overnight cultures inoculated to an OD 0.05 in prewarmed LB broth containing tetracycline. One-millilitre culture samples were harvested by centrifugation, and the pellets frozen at −20 °C.

To determine relative light units (RLU), pellets were thawed briefly and resuspended in PBS (pH 7.4) to an

OD of either 10 or 1, depending on induction levels. Aliquots of the cell suspensions were then mixed with equal aliquots of Luciferase Assay System substrate (Promega), and luminescence was measured for 15 s after a delay of 3 s on a Turner Designs TD-20/20 luminometer (Promega) as previously described (Dengler *et al.*, 2011).

Bacitracin gradient plates and Etests

Qualitative differences in resistance levels for bacitracin (from *Bacillus licheniformis*, Sigma) were compared using antibiotic gradient plates as previously described (Hübscher *et al.*, 2009). LB medium was supplemented with $ZnCl_2$ (25 μg mL^{-1}), and plates were incubated at 37 °C for 48 h. Bacitracin minimum inhibitory concentrations (MIC) were detected by Etest (Bio-Mérieux) on Müller-Hinton plates swabbed with an inoculum of 0.5 McFarland and incubated at 37°C for 24 h.

Growth under subinhibitory concentrations of tunicamycin

Overnight cultures were diluted to OD 0.05 in LB media containing 0.05 μg mL^{-1} tunicamycin (AG Scientifics). OD measurements were taken hourly for 8 h.

Preparation and quantification of WTA

Cell walls and WTA were prepared as previously described (Majcherczyk *et al.*, 2003). The amount of WTA was indirectly quantified by determination of the cell wall phosphorus content (Ames & Dubin, 1960). Experiments were performed two to four times with three technical replicates per sample.

Results and discussion

Deletion of LCP proteins leads to increased VraSR-dependent basal expression of the CWSS

LCP proteins are essential for optimal cell separation (Over *et al.*, 2011). The severe cell division defects of double and triple LCP mutants resemble those resulting from the depletion of essential peptidoglycan biosynthesis enzymes or inhibition of WTA synthesis, which both trigger VraSR signal transduction and induction of the CWSS (Gardete *et al.*, 2006; Sobral *et al.*, 2007; Balibar *et al.*, 2009; Blake *et al.*, 2009; Campbell *et al.*, 2012). The most sensitive indicator of staphylococcal CWSS activation is the *sas016* gene, as demonstrated previously in Northern blot, promoter-luciferase fusion and microarray studies; however, its function is still unknown (McAleese *et al.*, 2006; Dengler *et al.*, 2011). We therefore determined the basal CWSS transcription levels of single, double and triple LCP mutants and compared them to those of the parent strain MSSA1112 using a probe against the CWSS gene *sas016*. Northern blots showed that *sas016* transcription was detectably higher in single LCP mutants than in the wild type, with highest levels of transcription in the *Δsa0908* mutant (Fig. 1a). Transcript levels were further increased in double LCP mutants, *Δsa0908/msrR*, *Δsa2103/msrR* and *Δsa2103/sa0908*, and were extremely high in the LCP triple mutant (Fig. 1a).

To compare and quantify CWSS expression at different growth stages, a promoter-luciferase reporter construct containing the *sas016* promoter (p*sas016*$_p$-*luc*+) was used as previously described (McCallum *et al.*, 2011). Figure 1b shows the luciferase activity levels measured in relative light units (RLU) in the wild type and LCP mutant strains at the time points indicated. The right graph shows the corresponding OD values of the cultures at each sampling point. To confirm patterns of CWSS upregulation, expression of the autoregulatory *vra* promoter from the *vraSR* operon was also measured, using the promoter-luciferase fusion p*vra*$_p$-*luc*+ (Supporting information, Fig. S1). Both constructs, p*sas016*$_p$-*luc*+ and p*vra*$_p$-*luc*+ displayed very similar luciferase activity profiles, with expression from the *vraSR* operon promoter being consistently lower than that from the *sas016* promoter, reflecting differences in promoter activity that were observed in previous transcriptional analyses of the CWSS (McAleese *et al.*, 2006). In all strains tested, the activity increased during exponential growth and decreased again as cells entered stationary phase, with maximum luciferase activity levels reached in late exponential growth, at around 4.5 h.

Luciferase activity profiles corresponded closely to the results from Northern blots (Fig. 1a). Expression was reproducibly higher in LCP single mutants than in the parent MSSA1112, with up to twofold increases in *Δsa2103* and *ΔmsrR* mutants and a larger, up to sixfold increase, in *Δsa0908*. The luciferase expression from the *sas016* promoter increased further in the double LCP mutants with the highest expression levels seen in *Δsa2103/sa0908* and comparable levels in *Δsa0908/msrR* and *Δsa2103/msrR*. The most dramatic increase was apparent in the triple mutant, where expression levels were up to 250-fold higher than in the wild type, similar to levels reached after antibiotic stress (Fig. 1e). Activity peaked slightly later in some mutants, possibly reflecting minor differences in growth dynamics.

To verify that increased CWSS expression was VraSR dependent, a VraR mutation was introduced into the wild type strain MSSA1112 and all single and double mutants. The VraR mutation could not be introduced into the triple mutant, probably due to its cell separation

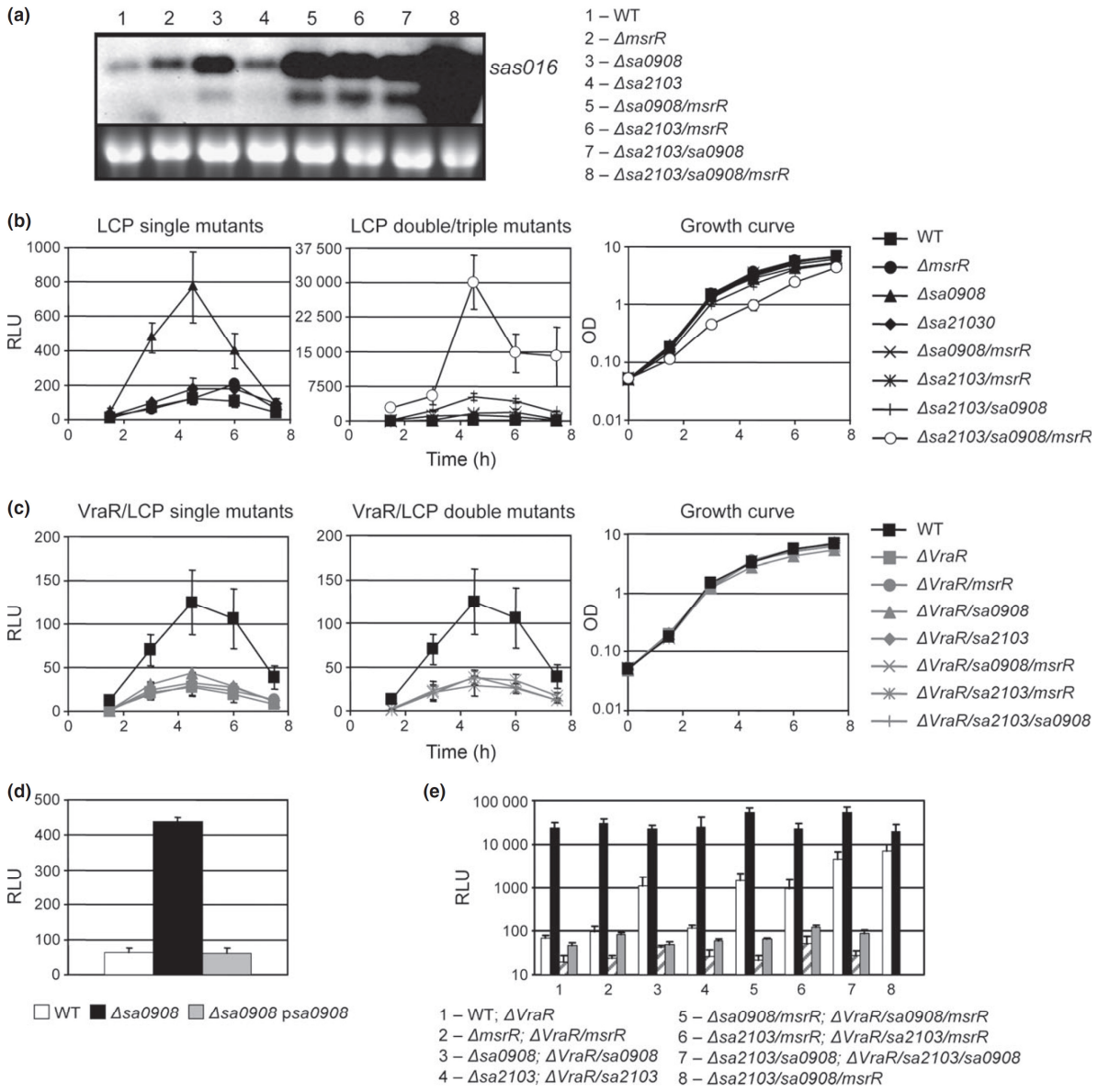

Fig. 1. CWSS expression in LCP and VraR/LCP mutant strains. (a) Northern blot analysis showing the expression of the CWSS gene *sas016* in LCP mutants. (b and c) Luciferase activities measured from reporter construct p*sas016*$_p$-*luc*+ in LCP mutants (b) and in VraR/LCP mutants (c). Values shown indicate the RLU measured in each of the strains at the different growth stages indicated. Left, single LCP or VraR/LCP mutants; middle row, LCP or VraR/LCP double and triple mutants; right, corresponding OD values of the cultures at each sampling point for all strains. Samples were taken at 1.5-h intervals for up to 7.5 h. The RLU scales of the different graphs were adjusted to appropriate ranges for visualizing strain-dependent differences. Average values and standard deviations from three independent experiments are shown. (d) Complementation of the *Δsa0908* mutant strain by introducing *sa0908 in trans*. RLUs were measured from strains containing the reporter construct p*sas016*$_p$-*luc*+ that were harvested between OD 0.6 and 0.8. Values shown represent the averages and standard deviations from three independent experiments. (e) Luciferase activities of LCP and VraR/LCP mutants with and without oxacillin (10 μg mL^{-1}) induction. Cultures were grown to OD 1.5–1.8 before being split into two prewarmed flasks, one culture was induced with oxacillin and the other left uninduced, and cultures were grown for a further 30 min before samples were harvested. RLU values are shown on a logarithmic scale and represent the averages and standard deviations from three independent experiments. Untreated LCP mutants are shown in white, treated LCP mutants in black, untreated VraR/LCP mutants in grey/white hatched and treated VraR/LCP mutants in grey.

defects and temperature sensitivity (Over *et al.*, 2011). Expression of the CWSS was measured over growth in the VraR/LCP mutants using p*sas016*$_p$-*luc*+. In all *ΔVraR* mutants, CWSS expression levels dropped clearly below wild type values (Fig. 1c). The minor differences in expression between all VraR/LCP mutants and MSSA1112*ΔVraR*, indicates that the increased basal CWSS expression levels in LCP mutants were VraSR dependent.

Complementation of *Δsa0908*, the single mutant with the strongest effect on CWSS expression, by re-introduction of *sa0908 in trans*, reduced luciferase activity back to wild type levels (Fig. 1d), demonstrating that differences in CWSS activity were directly linked to the LCP mutations.

LCP mutants are still responsive to cell wall stress

As the CWSS was already inherently activated to varying degrees in the absence of external stress in growing LCP mutants, we tested their potential to react to an external cell wall stress. Luciferase activity from p*sas016*$_p$-*luc*+ was measured in exponentially growing LCP and VraR/LCP mutants exposed to oxacillin for 30 min (Fig. 1e). Basal transcription levels were again increased in uninduced LCP mutants. Expression was still strongly induced by oxacillin stress in the single and double LCP mutants. Expression in the untreated LCP triple mutant appeared to already be close to the maximum level, as it only increased approximately twofold upon oxacillin stress (Fig. 1e).

Identification of promoter regions involved in CWSS induction

Two VraR-binding sites have been identified in the promoter of the *vraSR* operon with a tail to tail tandem repeat motif ACT(X)$_n$AGT (X = A, C, T or G; *n* = 1–3; Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012). They are involved in the fine tuning of the VraR-dependent expression of the CWSS and have different affinities for VraR or phosphorylated VraR (Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012). VraR-binding sites in other CWSS promoters have so far only been studied *in silico*. A 16-bp palindromic sequence **T**CAGHCTnnAGDCTG**A** (H = A, T, C; D = A, T, G), deduced from the VraR homologue CesR in *L. lactis* (Martinez *et al.*, 2007) and partially overlapping the motif identified by Belcheva *et al.*, is present in the promoters of 26 VraSR-dependent genes of the *S. aureus* N315 genome (Martinez *et al.*, 2007). As we found the induction levels of the three LCP genes and of the highly induced CWSS gene *sas016* to vary over a wide range, we analysed their specific VraR-binding motifs. The transcriptional start sites of *msrR*, *sa0908* and *sa2103* are known (Rossi *et al.*, 2003; Over *et al.*, 2011), and the transcriptional start site of *sas016* was determined by primer extension to be 29-nt upstream of the ATG (data not shown). Potential VraR-binding sites were predicted in all four promoters investigated in this study, based on previously published motifs (Martinez *et al.*, 2007; Belcheva & Golemi-Kotra, 2008; Belcheva *et al.*, 2012). These sequences were then disrupted and/or deleted in the promoter regions of luciferase reporter gene constructs (Fig. 2). Disruption of the predicted motifs decreased basal expression levels and largely abolished induction by oxacillin (Fig. 2). In all four promoters, the regions essential for induction were located close to the −35 boxes. The promoter of *sas016* contained a second region that was found to be essential for full induction. The presence of two VraR-binding sites could contribute to the extremely high induction levels of *sas016*. Alignment of the nucleotide sequences from the VraR-binding regions identified here revealed no obvious consensus sequence. The high-affinity VraR-binding region in the *vraSR* operon promoter (Belcheva *et al.*, 2012) and the *tcaA* promoter region required for induction (McCallum *et al.*, 2007) were both also in close proximity to their respective −35 box. The *msrR* promoter region needed for induction corresponded to the CesR-like motif identified *in silico* by Martinez *et al.* (2007; Fig. 2); however, deletion of the suggested CesR-binding region for *sa0908* did not affect transcription (data not shown). For the promoters of *sas016* and *sa2103*, no CesR-like binding sites were previously predicted (Martinez *et al.*, 2007); however, the VraR-binding sites identified here both contained potential CesR-like sequences. To create a reliable VraR-binding consensus for *S. aureus* CWSS gene induction, detailed promoter analysis of more VraSR-dependent genes is required. The trend, however, seems to involve sequences with a close proximity to the −35 box of the CWSS gene promoter.

Bacitracin hypersensitivity of the LCP triple mutant

Bacitracin inhibits the recycling of the universal undecaprenyl phosphate lipid carrier by preventing dephosphorylation of the undecaprenyl pyrophosphate (Stone & Strominger, 1971; Qi *et al.*, 2008). Kawai *et al.* (2011) recently suggested that LCP proteins transfer WTAs and other anionic polymers from the lipid carrier to the cell wall peptidoglycan in *B. subtilis*. Comparative growth of LCP mutants on bacitracin gradient plates showed that the LCP triple mutant was highly susceptible (Fig. 3a). The bacitracin MIC of the triple mutant was 4 μg mL^{-1}

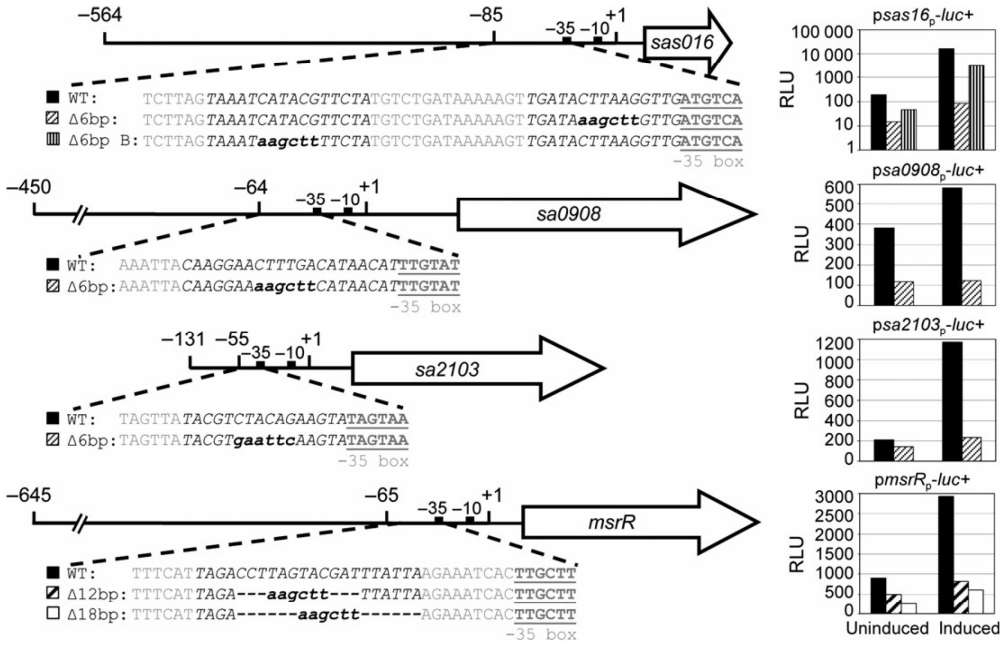

Fig. 2. Analysis of predicted VraR-binding sites in the *sas016*, *msrR*, *sa0908* and *sa2103* promoters. Nucleotide sequences of promoter regions and introduced promoter mutations are shown, together with their corresponding luciferase activities when fused to the luciferase gene and introduced into *Staphylococcus aureus* strain RN4220. RLU of the promoter constructs were measured with and without 30 min of induction with 10 μg mL^{-1} oxacillin. Cultures were grown to OD 0.5–0.8 before splitting into two prewarmed flasks comprising the uninduced and oxacillin-induced samples. Predicted VraR-binding regions are shown in black italic capitals; −35 boxes in bold grey underlined capitals; restriction sites in bold italic lowercase letters; deleted regions are indicated by a dashed line. Representative results from three independent experiments are shown.

compared to 32 μg mL^{-1} for wild type and all LCP single and double mutants. The hyper susceptibility of the LCP triple mutant to bacitracin could therefore be due to an additional shortage of the lipid carriers caused by the lack of the putative WTA ligase function of LCP proteins.

Deletion of all three LCP proteins in *S. aureus* depletes WTA content

In line with the proposed function of LCP proteins, previous studies showed a decrease in the WTA content of LCP mutants in different species (Hübscher *et al.*, 2008; Kawai *et al.*, 2011). Therefore, we analysed the WTA content of LCP single mutants and the triple mutant in *S. aureus*, via detection of the cell wall phosphorus content (Ames & Dubin, 1960). The previously described decrease in the WTA content of the *ΔmrsR* mutant (Hübscher *et al.*, 2009) could be confirmed here, and the WTA contents of the *Δsa0908* and *Δsa2103* mutants were decreased to 62% and 95% of the wild type level, respectively (Fig. 3b). An almost complete depletion of WTA was observed in the triple LCP mutant, with cell wall phosphorus content down to 2% of the wild type. Re-introduction of single LCP genes into the triple mutant restored WTA levels to 94%, 81% and 69% of wild type levels for *sa2103*, *msrR* and *sa0908*, respectively. The capacity of all LCP proteins to restore the WTA content to a certain degree confirmed a partial functional redundancy that has been shown for other phenotypes such as growth defects, beta-lactam resistance, biofilm formation and self-agglutination (Over *et al.*, 2011). The very low WTA content of the LCP triple mutant confirmed that LCP proteins in *S. aureus* have an essential function in WTA loading of the cell wall.

TarO (TagO) inhibition can partially complement the growth defect of the *S. aureus* LCP triple mutant

The three LCP genes in *B. subitlis* are conditionally essential, meaning that an LCP triple mutant in *B. subtilis* is only viable when *tagO* (*tarO*) is also deleted, thereby preventing the flux of precursors into the WTA synthesis pathway (Kawai *et al.* 2011). The effect of TarO (TagO) inhibition on the LCP triple mutant was tested to detect a possible connection between LCP proteins with WTA

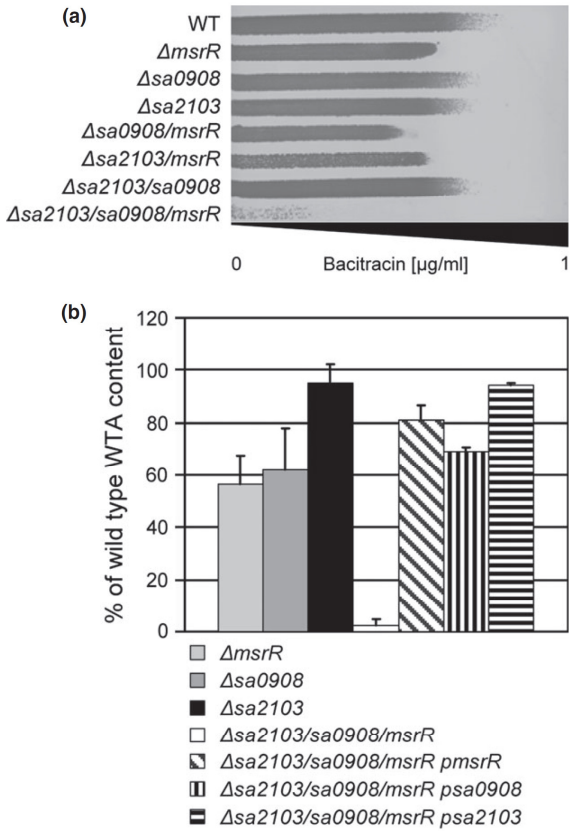

Fig. 3. Bacitracin susceptibility and phosphorus content of the cell wall (WTA content) of LCP mutants. (a) Bacitracin gradient plates of LCP mutants. MICs of bacitracin were detected by Etest (BioMérieux): wild type MSSA1112 and LCP single and double mutants all had MICs of 32 μg mL^{-1}, LCP triple mutant had an MIC of 4 μg mL^{-1}. (b) Relative levels of WTA contents are shown as percentages of wild type MSSA1112 content, determined indirectly by detecting the phosphorus content of the cell wall for LCP single mutants, the LCP triple mutant and complemented triple mutants. The experiment was performed two to four times with three technical replicates per sample. The average of the absolute values for the wild type was 0.81 ± 0.04 μmol phosphorus per mg cell wall.

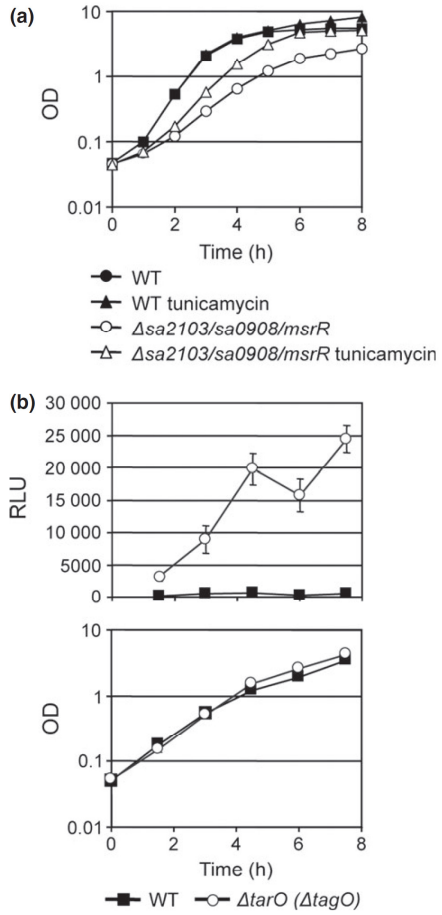

Fig. 4. Growth of the LCP triple mutant under subinhibitory concentrations of tunicamycin and CWSS expression in a *tarO* (*tagO*) mutant strain. (a) Growth of the LCP triple mutant and wild type MSSA1112 with and without tunicamycin (0.05 μg mL^{-1}). Average values and standard deviations from three independent experiments are shown. (b) Luciferase activity, in RLU, at different growth stages in the wild type strain SA113 and the SA113*ΔtarO* mutant strain, measured from reporter construct p*sas016*$_P$-*luc*+. Upper graph shows luciferase measurements and lower graph, the corresponding OD values of the cultures at each sampling point for all strains. Average values and standard deviations from three independent experiments are shown.

synthesis or assembly in *S. aureus*, as found for *B. subtilis* (Kawai *et al.*, 2011). Subinhibitory concentrations of tunicamycin, which are known to inhibit TarO (TagO; Campbell *et al.*, 2011), could partially complement the growth defect of the LCP triple mutant (Fig. 4a). The minimal doubling time of the triple mutant decreased from 49 ± 2 to 34 ± 2 min upon tunicamycin treatment. Inhibition of TarO in the wild type did not significantly affect the minimal doubling time of 25 ± 0.6 min but reduced the maximal OD reached after 8 h of growth from 8.2 to 5.5. This result supports an involvement of LCP proteins in a late step of WTA synthesis in *S. aureus*. As LCP proteins in *B. subtilis* are essential, it could be that the staphylococcal LCP triple mutant is only viable because of compensatory mutations, which remains to be verified. However, it is also possible that the functions of LCP proteins in *S. aureus* are not identical to those in *B. subtilis*, because differences have been found in the WTA synthesis pathways of these closely related bacteria (Brown *et al.*, 2010). Also, in contrast to *S. aureus*, WTA-deficient strains in *B. subtilis* have significantly decreased growth rates and lost their rod shape, indicating potential

differences in the roles of WTA ligases in *B. subtilis* and *S. aureus* cell division (Weidenmaier *et al.*, 2004; D'Elia *et al.*, 2006).

Deletion of *tarO* (*tagO*) induces the CWSS

Measurement of CWSS expression in an *S. aureus* SA113*ΔtarO* (*ΔtagO*) mutant (Weidenmaier *et al.*, 2004), with the reporter plasmid p*sas016*$_p$-*luc*+, revealed that inhibition of the first step of WTA synthesis induces the CWSS (Fig. 4b). This result is in conflict to the observations by Campbell *et al.*, (2011) who showed that inhibition of TarO (TagO) by subinhibitory concentrations of tunicamycin does not induce the CWSS. They suggested that CWSS induction is triggered by the sequestration of the lipid carrier rather than WTA deficiency (Campbell *et al.*, 2011, 2012). However, our analysis of the *tarO* (*tagO*) mutant indicates that further research is required to reveal the actual trigger of CWSS induction.

Conclusions

Deletion of LCP proteins increased basal expression levels of CWSS genes via the VraSR two-component system. The LCP triple mutant showed very high basal expression of the CWSS, close to levels triggered by antibiotic stress. The LCP double and single mutants, however, still responded to cell wall stress by further upregulating the CWSS.

Promoter regions required for VraR-dependent induction of the LCP genes and *sas016* shared low overall nucleotide similarity, but all contained fragments of the predicted CesR-like binding consensus or the VraR-binding motif of the *vraSR* operon and all were in close proximity to the −35 box of the gene's promoter.

Hyper susceptibility of the triple mutant to bacitracin, the virtual absence of WTA and partial restoration of WTA levels by complementation with each of the single LCP proteins, as well partial complementation of its growth defect by TarO (TagO) inhibition, support the hypothesis that *S. aureus* LCP proteins have WTA ligase functions, as suggested by Kawai and colleagues for *B. subtilis* (Kawai *et al.*, 2011).

An enzymatic analysis of all three LCP proteins will be required to confirm their specific WTA ligase functions, substrates and products.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CWSS expression in LCP mutant strains measured with pvra_p-*luc*+.

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Figure S1

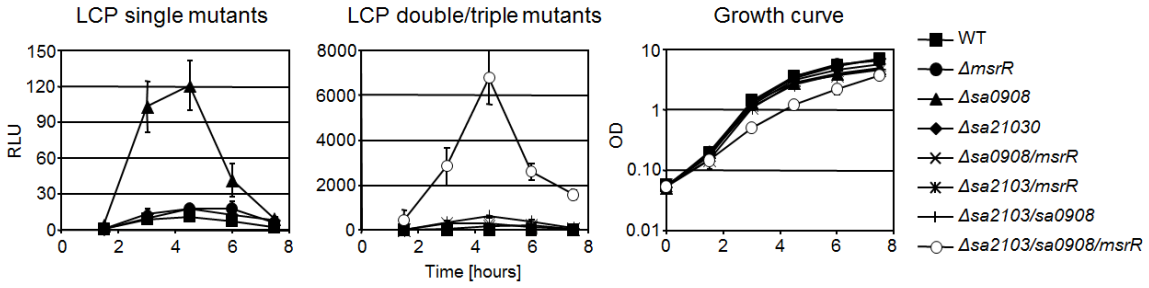

Figure S1 - CWSS expression in LCP mutant strains measured with p*vra*$_p$*-luc*+.
Luciferase activity, in relative light units (RLU), at different growth stages of LCP mutants from reporter construct p*vra*$_p$*-luc*+. Left, single LCP mutants; middle, LCP double and triple mutants; right corresponding OD values of the cultures at each sampling point for all strains. Samples were taken at 1.5 h intervals for 7.5 h. Different RLU scales are used for different graphs in order to accommodate the ranges of RLU measured and highlight strain-dependent differences. Average values and standard deviations from three independent experiments are shown.

Author contributions:

VD participated in design of the study, performed most of the experimental work and drafted the manuscript. PSM, PM and BBB participated in the design of the study and in writing of the manuscript. PK, RH, PAM and JF performed experimental work and helped with interpretation of results. SF and SBS performed preliminary experiments crucial for the study. NM participated in the design and coordination of the study, in writing of the manuscript and in experimental work.

3.3. Project III:

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PLOS ONE

Mutation in the C-Di-AMP Cyclase *dacA* Affects Fitness and Resistance of Methicillin Resistant *Staphylococcus aureus*

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Abstract

Faster growing and more virulent strains of methicillin resistant *Staphylococcus aureus* (MRSA) are increasingly displacing highly resistant MRSA. Elevated fitness in these MRSA is often accompanied by decreased and heterogeneous levels of methicillin resistance; however, the mechanisms for this phenomenon are not yet fully understood. Whole genome sequencing was used to investigate the genetic basis of this apparent correlation, in an isogenic MRSA strain pair that differed in methicillin resistance levels and fitness, with respect to growth rate. Sequencing revealed only one single nucleotide polymorphism (SNP) in the diadenylate cyclase gene *dacA* in the faster growing but less resistant strain. Diadenylate cyclases were recently discovered to synthesize the new second messenger cyclic diadenosine monophosphate (c-di-AMP). Introduction of this mutation into the highly resistant but slower growing strain reduced resistance and increased its growth rate, suggesting a direct connection between the *dacA* mutation and the phenotypic differences of these strains. Quantification of cellular c-di-AMP revealed that the *dacA* mutation decreased c-di-AMP levels resulting in reduced autolysis, increased salt tolerance and a reduction in the basal expression of the cell wall stress stimulon. These results indicate that c-di-AMP affects cell envelope-related signalling in *S. aureus*. The influence of c-di-AMP on growth rate and methicillin resistance in MRSA indicate that altering c-di-AMP levels could be a mechanism by which MRSA strains can increase their fitness levels by reducing their methicillin resistance levels.

Citation: Dengler V, McCallum N, Kiefer P, Christen P, Patrignani A, et al. (2013) Mutation in the C-Di-AMP Cyclase *dacA* Affects Fitness and Resistance of Methicillin Resistant *Staphylococcus aureus*. PLoS ONE 8(8): e73512. doi:10.1371/journal.pone.0073512

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Introduction

Infections with methicillin resistant *Staphylococcus aureus* (MRSA) have severely impaired treatment outcomes and are cost intensive for the healthcare system. MRSA contain a staphylococcal cassette chromosome *mec* (SCC*mec*) element harbouring the *mecA* gene that codes for an alternative penicillin binding protein, PBP2a, which confers resistance to beta-lactams, the antibiotic class of first choice for treating *S. aureus* infections. Initially, MRSA were restricted to healthcare settings with high antibiotic pressure. These healthcare-associated (HA)-MRSA strains generally displayed low heterogeneous resistance profiles, whereby only small subpopulations could survive at high beta-lactam concentrations. Over time, HA-MRSA strains possessing very high beta-lactam minimum inhibitory concentrations (MICs) emerged, such as E-MRSA 16 (ST32-MRSA-II) or the Iberian clone (ST247-MRSA-I), which successfully spread in hospitals all over the world (for reviews see [1,2]). Over the last fifteen years MRSA have started to spread in the community [3]. These so-called community-acquired (CA)-MRSA are characteristically fitter and more virulent than HA-MRSA strains (for reviews see [4,5]). CA-MRSA strains, such as USA300, are capable of infecting healthy individuals without obvious risk factors [3]. CA-MRSA typically have relatively low oxacillin MICs, but their heterogeneous resistance profiles include higher-resistant subpopulations of bacteria that can cause treatment failure. CA-MRSA-like clones are increasingly displacing conventional, previously successful, HA-MRSA clones in hospitals [6].

Methicillin resistance does not only depend on PBP2a production but it is also affected by the genetic background of a strain [7] and factors influencing methicillin resistance, previously called *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) factors [8,9,10]. These factors are often directly or indirectly involved in cell envelope biosynthesis and turnover. Examples of *fem*/*aux* factors linked to the cell envelope include cell wall biosynthesis enzymes like GlmM [11], MurE [12], MurF [13], FemABX [14], PBP2 [15], PBP4 [16]; and wall teichoic acid biosynthesis enzymes including TagO/TarO [17], the *dlt* operon [18] and the wall teichoic acid ligase MsrR [19,20]. *Fem*/*aux* factors indirectly connected or with no obvious connection to the cell envelope include regulators like SigB [21], SpoVG [22], *agr* [23], SarA [23], XdrA [24], CcpA [25], SecDF [26] or two component systems like VraSR [27,28].

Recently, a new second messenger, cyclic diadenosine monophosphate (c-di-AMP) was shown to influence methicillin resistance in *S. aureus* [29]. Second messengers are small molecules such as the nucleotides cAMP, cGMP, c-di-GMP or (p)ppGpp that regulate various cellular processes including virulence, biofilm formation, motility and the cell cycle. Levels of the nucleotide (p)ppGpp, involved in the stringent response, were also recently discovered to influence beta-lactam resistance [30]. Regulation by nucleotides can occur at transcriptional, translational and post translational levels (for reviews see [31,32]). c-di-AMP is synthesized by condensation of two ATP molecules by diadenylate cyclase domain (DAC) proteins and was first identified by Witte *et al.* in *Bacillus subtilis* in 2008 [33]. In contrast to *B. subtilis*, which has three different proteins with DAC domains, *S. aureus* possesses only one DAC domain protein, DacA. DacA is claimed to be essential and shows a similar structure to the *B. subtilis* DAC domain protein CdaA (previously called YbbP) containing a transmembrane domain and the DAC domain [29]. Additionally, the genetic regions of *S. aureus* and *B. subtilis* have a similar organisation forming the three-gene operons *dacA-ybbR-glmM* and *cdaA-cdaR-glmM*, respectively, including the phosphoglucosamine mutase *glmM and* the recently identified c-di-AMP regulator *cdaR* or its *S. aureus* homologue *ybbR* [11,34]. However, in *B. subtilis* the glucosamine-6-phosphate synthase gene *glmS* is located directly downstream of *glmM* and forms an additional large transcript *cdaA-ybbR-glmM-glmS* [34]. In contrast, *glmS* in *S. aureus* is separated from *glmM* by about 14 kb, which contains the cell wall associated protein *fmtB* (also called *mrp*) and mannitol synthesis and export genes [35]. A larger transcript including *fmtB* but not *glmS* has been detected in *S. aureus (dacA-ybbR-glmM-fmtB)* [36]. In *B. subtilis* the protein encoded directly upstream of the diadenylate cyclase, CdaR (previously called YbbR), is a c-di-AMP synthase regulator which was found to specifically stimulate c-di-AMP production by CdaA [34]. Mehne and colleagues suggested that the same positive regulation could occur by YbbR of *S. aureus* since DacA and CdaA share several common features, as described above. The cellular level of c-di-AMP is further influenced by degradation of c-di-AMP to 5'-pApA by the phosphodiesterase GdpP [29,37]. In several studies, mutations in *gdpP* were found to increase resistance or tolerance to beta-lactam antibiotics in *B. subtilis*, *Listeria monocytogenes* and *S. aureus* by as yet unknown mechanisms [29,38,39,40,41,42]. Besides antibiotic resistance, c-di-AMP affects cell envelope homeostasis in both *B. subtilis* and *S. aureus* [29,40]. In *S. aureus* a 15-fold increased c-di-AMP level led to increased peptidoglycan cross-linking and could compensate for the absence of lipoteichoic acids [29]. Interestingly, c-di-AMP secreted from *L. monocytogenes* triggers a host type I interferon response; a c-di-AMP dependent type I interferon response was also detected in the Gram-negative *Chlamydia trachomatis* [43,44]. Furthermore, a *gdpP* mutation in *Lactococcus lactis* led to salt hypersensitivity and heat resistance [45].

Very recently the first c-di-AMP receptors were identified [46,47]. One is the TetR-like transcription factor DarR of *Mycobacterium smegmatis* [47] and four receptor proteins were identified in *S. aureus*; the potassium transporter-gating component KtrA, a predicted cation/proton antiporter CpaA, a PII-like signal transduction protein PstA and a histidine kinase KdpD [46]. However, it remains unclear how these c-di-AMP receptors influence cell envelope homeostasis and beta-lactam resistance, indicating that there might be more receptors.

Even though there has been extensive research on methicillin resistance, the mechanisms governing heterogeneous and homogeneous resistance in MRSA are not completely understood yet. In our study we analysed an isogenic MRSA strain pair differing in fitness and methicillin resistance levels. Whole genome sequencing identified only one single nucleotide polymorphism (SNP) which was in the diadenylate cyclase gene *dacA*. Markerless allelic replacement was used to confirm that this mutation was responsible for the transition from homogeneous to heterogeneous resistance and the associated faster growth rate. Quantification of cellular c-di-AMP levels indicated that phenotypic changes were caused by decreased c-di-AMP, due to the *dacA*-SNP.

Materials and Methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA), shaking at 180 rpm with a 1:5 culture to air ratio, or on LB agar plates unless indicated differently. Optical density (OD) was measured at 600 nm. Media were supplemented with the following antibiotics when appropriate: 10 µg/ml tetracycline (Sigma, St. Lousis, MO, USA), 10 µg/ml chloramphenicol (Sigma), 100 µg/ml ampicillin (Sigma), 200 ng/ml anhydrotetracycline (Sigma) or various concentrations of oxacillin (InfectoPharm, Heppenheim, Germany).

Whole genome sequencing and data analysis

Genome sequencing of strains RA120 and ME51 was performed by GATC Biotech AG (Konstanz, Germany) on an Illumina HiSeq 2000 (Illumina Inc., Saffron Walden, United Kingdom). Mapping of the 31-bp reads of a 200-bp paired-end library against the reference genome NCTC8325 and SNP calling was performed with the CLC Genomics workbench 4.8 (CLC Bio, Aarhus, Denmark), resulting in 63-fold mean depth coverage for RA120 (6'135'387 reads) and 83-fold mean depth coverage for ME51 (7'658'818 reads). The parent strain BB255 was sequenced using a PacBio RS SMRT sequencer (Pacific Biosciences Inc., Menlo Park, CA, USA) at the Functional Genomics Center Zurich. DNA from standard phenol/chloroform/isoamyl alcohol extractions was purified over DNeasy Mini Spin Columns (Qiagen, Düsseldorf, Germany). Two 250-bp libraries and one 6-kb library were prepared with the PacBio C1 chemistry kits provided by Pacific Biosciences according to the manufacturers' recommendations. The library was quality inspected and quantified on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and on a Qubit Fluorometer (Life technologies Europe, Zug, Switzerland), respectively. After the run, sequencing reports were generated via the SMRT portal to assess the adapter dimer contamination, the sample loading efficiency, the obtained average read length and the number of filtered sub-reads. Mapping of the sub-reads against the reference genome NCTC8325 and SNP calling was performed automatically via the SMRT portal and confirmed using CLC Genomics Workbench 4.8. A 174-fold mean depth coverage from 1'978'548 reads with an average read length of 199 bp from the 250-bp libraries and 126'945 reads with an average read length of 981 bp including reads reaching up to 17'000 bp from the 6-kb library were achieved. The sequencing data was submitted to the European Nucleotide Archive (ENA) under the study accession number PRJEB400 and the sample accession numbers ERS251419 (BB255), ERS251420 (RA120) and ERS251421 (ME51).

Resequencing and construction of mutant strains

The identified SNP in *dacA* was confirmed by Sanger sequencing with the primers 2407.F (TTATTTCACACCTTTCTTTTGAAAG) and 2407.R (ATGGATTTTTCCAACTTTTTTCAAA) at Mircosynth (Balgach, Switzerland).

Table 1. Strains and plasmids.

Strain or plasmid name	Relevant genotype and/or phenotype	Source or reference
Strains		
S. aureus		
RN4220	Restriction-deficient derivative of NCTC 8325-4	[68]
BB255	NCTC8325 derivative, FK268 cured from plasmid pI524 by ethidium bromide treatment, ST8, Mc^S	[53]
RA120	BB255 containing SCC*mec* type I, Mc^R	[54]
ME51	RA120 derivative with increased fitness isolated from competition experiments, mutation in DacA (Gly206Ser), Mc^R	[54]
COL	Clinical HA-MRSA, ST250, Mc^R	[9]
BB255::*dacA-SNP*	BB255 with mutation in DacA (Gly206Ser), Mc^S	This study
RA120::*dacA*-SNP	RA120 with mutation in DacA (Gly206Ser), Mc^R	This study
COL::*dacA-SNP*	COL with mutation in DacA (Gly206Ser), Mc^R	This study
E. coli		
DH5α	F^- ϕ80d/acZΔM15 *recA1*	Invitrogen
Plasmids		
pKOR1	*S. aureus-E. coli* shuttle vector, *ori* pAMα1, *ori* ColE1, *E. coli* Am^r, *S. aureus* Cm^r	[48]
pKOR1-*dacA*-SNP	pKOR1 construct containing the *dacA* gene with 1000-bp upstream and 1020-bp downstream regions carrying a mutation leading to Gly206Ser substitution in DacA	This study
p*sas016*p-*luc*+	pBUS1 containing the *sas016* promoter-luciferase reporter gene fusion	[67]

Abbreviations: Am, ampicillin; Cm, chloramphenicol; Mc, methicillin; Tc, tetracycline; r, resistant; s, susceptible; ST, sequence type.
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The pKOR1 system developed by Bae & Schneewind was used to insert the mutation into *dacA* by homologous recombination [48]. The *dacA* gene, with additional 1000-bp upstream and 1020-bp downstream regions, was amplified from genomic DNA of strain ME51 using primers attB1-2407-upF (GGGG ACAAGTTTG-TACAAAAAAGCAGGCT CGACACCTCTTACTCCGTCT) and attB2-2407-downR (GGGG ACCACTTTGTACAA-GAAAGCTGGGT ACCAACAGCAATTAGATATG) and the fragment was recombined into the pKOR1 plasmid. Markerless allelic replacement was then performed as previously described [48].

Oxacillin population analysis profiles and determination of oxacillin minimum inhibitory concentration (MIC)

Antibiotic resistance profiles were determined by plating appropriate dilutions of an overnight culture, ranging from undiluted to 10^{-7}, on increasing concentrations of oxacillin. Plates were incubated at 37°C and colony forming units per ml (CFU/ml) were determined after 48 hours. Experiments were performed at least three times and representative data are shown. MICs were determined using Etest® strips (BioMérieux, Marcy l'Etoile, France) on Mueller-Hinton plates swabbed with an inoculum of 0.5 McFarland and incubated at 37°C for 24 h. MICs tests were performed at least three times.

Autolysis

Spontaneous and induced autolysis were determined as previously described [26]. Briefly, cells were grown to OD 0.7, washed with 0.85% NaCl and resuspended in 0.01 M Na-phosphate buffer pH 7. The OD was adjusted to 0.7, cultures were incubated at 37°C and decreases in OD were measured over time. For induced autolysis 0.01% Triton X-100 (Fluka, Buchs, Switzerland) was added to the phosphate buffer after washing. Values shown represent the means and standard deviation from three independent experiments. For statistical analysis of individual time points Student's *t*-tests were performed and for comparison of whole data sets two-way Anova was applied.

PBP2a and PBP4 Western blot

S. aureus membrane fractions were obtained as described by Quiblier *et al.* [26] and PBP2a Western blot analyses were performed as described by Ender at al. [49]. Briefly, cells were sampled by centrifugation and pellets were frozen in liquid nitrogen then resuspended and lysed with lysostaphin, lysozyme and DNase in SSM buffer (500 mM sucrose, 2 mM malate, 20 mM $MgCl_2$, pH 6.8). After five cycles of freezing and thawing the membrane fraction was obtained by centrifugation at 20'000 g and resuspended in glycerol buffer (30% glycerol, 150 mM NaCl, 25 mM TrisHCl, 1 mM $MgCl_2$, pH 7.5). Protein concentrations were measured by Bradford assay (BioRad, Hercules, CA, USA) and 10–20 µg of protein were separated on a SDS-7.5% PAGE for PBP2a Western blots and 80 µg of protein were separated on a SDS-12% PAGE for PBP4 Western blots. Gels were blotted onto PVDF-membranes (Immobilon-P, Milipore, Billerica, MA, USA) and the membranes were blocked with 5% milk powder and with 40 µg/ml human IgG (Calbiochem, Merck, Darmstadt, Germany). PBP2a was detected using PBP2a antibodies (1:20'000, Denka Seiken, Tokyo, Japan) and Horseradish Peroxidase-Conjugated (HRP) goat anti-mouse IgG (1:2500, Jackson ImmunoResearch, West Grove, PA, USA). PBP4 was detected with PBP4 antibodies (1:2000, [50]) and goat anti-rabbit IgG HPR (1:5000, Jackson ImmunoResearch). Signals were quantified using the AlphaInnotech imager software (Santa Clara, CA, USA), loading differences were corrected and values were given as a percentage of the signal intensity of strain RA120 with standard deviation. Student's *t*-test was applied to determine the significance of any differences. Western blot analyses were performed on three independent protein extractions.

Bocillin-FL staining of PBPs

Cell membrane fractions were extracted as described above. Eighty µg of proteins from membrane fractions were incubated for 30 min at 35°C with the fluorescent penicillin analogue Bocillin-FL (Invitrogen) as previously described [51]. Samples were separated by a SDS-7.5% PAGE and fluorescence was visualized with the FluorChem™ SP imaging system (AlphaInnotech).

NaCl tolerance

Overnight cultures of strains were transferred into a single row of a microtiter plate and serial 5-fold dilutions in 0.85% NaCl were prepared. Dilutions were replica-plated onto increasing NaCl concentrations using an automatic inoculator (Microtiter, Dynatech AG, Switzerland). All plates were incubated at 37°C for 48 h and three independent experiments were performed.

Luciferase assays

Luciferase measurements were performed as described earlier [19]. Briefly, cultures were inoculated at OD 0.05 and 1 ml samples were harvested by centrifugation after 1.5, 3, 4.5, 6 and 7.5 hours. Pellets were thawed and resuspended in PBS to OD 10. Cell suspensions were mixed with equal amounts of Luciferase Assay System substrate (Promega, Madison, WI, USA) and luminescence was measured on a Turner Designs TD-20/20 luminometer (Promega). For statistical analysis of individual time points and for comparison of whole data sets, Student's *t*-test and two-way Anova were applied, respectively.

Cell sampling for c-di-AMP quantification

Overnight cultures were diluted to OD 0.05 and grown to OD 2. Cells were harvested by filtration using 0.2 µm RC filters (Sartorius Stedium Biotech, Göttingen, Germany) collecting 3 ml culture per filter and two filters for each of the five replicates. Filters were washed once with 10 ml Braun water (37°C, B. Braun Meslungen AG, Meslungen, Germany) and directly transferred into 12 ml of pre-cooled quenching solution (–20°C, 60% acetonitril (Sigma, CHROMASOLV® Plus), 20% methanol (Sigma), 20% 0.5 M formic acid). Three hundred µl of 0.5 µM c-di-GMP (Biolog, Bremen, Germany) solution were added as an internal standard and the solution was sonicated four times for 20 seconds before it was snap frozen in liquid nitrogen and lyophilised.

c-di-AMP analysis by LC-MS

Analyses were performed with a Rheos 2200 HPLC system (Flux Instruments, Basel, Switzerland) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with an electrospray ionisation probe. c-di-AMP esters were analyzed by a method previously used for online solid phase extraction (SPE) and LC-MS analysis of CoA esters [52] with the exception of the MS analysis which was optimized for ionization of c-di-AMP (for parameters see below). Besides desalting, the method allowed complete removal of polar compounds including mono-nucleotides from cell extracts and samples could be injected at higher concentration. To perform online SPE of c-di-AMP and c-di-GMP prior to LC separation two C18 analytical columns (Gemini 50×2.0 mm and 100×2.0 mm, particle size 3 µm; Phenomenex, Torrance, CA, USA) were used. Flow rate was 220 µl/min, solvent A was 50 mM formic acid adjusted to pH 8.1 with NH_4OH and solvent B was methanol. The injection volume was 10 µl. For online desalting, samples were loaded on 50×2.0 mm C18 column and the sample was washed on column for 5 min with 100% solvent A. During SPE the short column was connected to waste via a 6-port-valve and the 100×2.0 mm column was equilibrated with solvent A by an additional pump. After desalting both columns were connected in series and the following gradient of B was applied to separate c-di-AMP: 5 min, 5%; 15 min, 23%; 25 min, 80%; 27 min, 80%. The LC-MS system was equilibrated for 6 min at initial elution conditions between two successive analyses. The LC was coupled to the mass spectrometer. Sheath gas flow rate was 40, auxiliary gas flow rate was 30, tube lens was –93 V, capillary voltage was –35 V, and ion spray voltage was –4.0 kV. MS analysis was done in the negative FTMS mode at a resolution of 60,000 (m/z 400).

Quantification of c-di-AMP

c-di-AMP was quantified using c-di-GMP as an internal standard to compensate for loss during sample preparation. Though c-di-GMP and c-di-AMP are chemically very similar, they do not co-elute and other compounds present in the sample can influence electrospray ionization of compounds. Therefore, the area response factors of the two compounds were determined in a series of standard solutions relative to the series of *S. aureus* cell extracts spiked with standards. The slope of a linear calibration curve (y = m*x+b) was determined for both compounds in standard and in cell extracts and slope ratios ($m_{sample}/m_{standard}$) were calculated. Values were used to correct corresponding peak areas measured in *S. aureus* extracts. The detection limit of 32 nM c-di-AMP (corresponding to 0.3 pmol c-di-AMP on the column) was estimated from the intercept of the calibration curve.

Results and Discussion

Identification of mutation in the diadenylate cyclase gene *dacA*

In previous studies a highly and homogeneously resistant MRSA was constructed by introducing the SCC*mec* element from strain COL into the methicillin sensitive *S. aureus* (MSSA) strain BB255 [53,54]. Introduction of the resistance cassette caused a fitness cost and significantly reduced the growth rate of the resulting MRSA strain RA120. From this slow growing strain RA120, a faster growing variant, ME51, was isolated during a competitive growth experiment [54]. Rescue from the fitness burden was accompanied by reduced, heterogeneous resistance to oxacillin [54]; a phenotype observed in CA-MRSA strains [4]. Genome sequencing of these two MRSA strains, RA120 and ME51, revealed 80 SNPs or deletion/insertion polymorphisms (DIPs) present in both strains, compared to the published NCTC8325 sequence (GenBank accession CP000253). Therefore, the parent strain BB255 was subsequently sequenced, confirming that these SNPs were already present in BB255, which excluded the possibility that these genetic differences were connected to the introduction of the SCC*mec* element (complete Table S1). All 46 sequencing errors identified in the NCTC8325 sequence by Berscheid *et al.* were confirmed in BB255 [55]. Twelve SNPs and one DIP were likely to be additional sequencing errors in the published NCTC8325 sequence since they were present in all other published *S. aureus* genomes including the NCTC8325 derivative RN4220 [56] (Table 2, marked with a cross in column RN4220). Thus, 21 SNPs or DIPs were identified to be real differences between BB255 and other NCTC8325 derivatives (Table 2). These mutations could either have been naturally acquired over time or caused by the ethidium bromide treatment of the original NCTC8325 FK268 that was used to cure it from its plasmid to create strain BB255 [53].

The SCC*mec* element introduced from strain COL was identical in both strains RA120 and ME51, but had four SNPs and a

Table 2. SNPs and DIPs identified in BB255, RA120 and ME51.

Position[1]	Ref[2]	Seq[3]	Impact	Locus (SAOUHSC_)[1]	Description[4]	RN4220[5]
5286	G	A	Gly85Ser	00166	DNA gyrase, ATP hydrolyzing subunite B	
22181	C	A		Intergenic 00018/00019	Non-coding	x
47652	T	-		Intergenic 00044/00045	Non-coding	x
73564	G	T	Asn472Lys	00069	Partial *spa* gene for immunglobulin G binding protein A	
142255	G	T	Gly200Trp	00136	CHP, nitrate transport ATP-binding protein NrtD, Putative ABC transporter	
210632	T	A	Asp454Glu	00190	CHP, membrane domain of membrane-anchored glycerophosphoryl diester phosphodiesterase	
218932	C	T	Asp63Asn	00197	Putative acyl-CoA dehydrogenase domain protein	
230630	T	G	Silent	00209	Putative PTS system maltose-and glucose-specific EIICB component	
252494	G	A	Gly323Asp	00230	Two-component sensor histidine kinase LytS	
329229	T	C	Leu131Ser	00314	Possible transcriptional regulator MarR family, MATE family multi-antimicrobial extrusion protein	
433209	A	T		Intergenic 00434/00435	Non-coding	
523591	G	A	Glu431Lys	00524	DNA-directed RNA polymerase beta subunit	
541723	T	G		Intergenic 00535/00536	Non-coding	
541724	G	C		Intergenic 00535/00536	Non-coding	
649126	G	T	Silent	00661	CHP, putative lipase/esterase	x
841103	G	T	Silent	00877	Iron-sulphur cluster assembly accessory protein	x
841139	G	T	Silent	00877	Iron-sulphur cluster assembly accessory protein	x
980692	C	T	Pro301Leu	01009	Phosphoribosylaminoimi-dazole carboxylase, ATPase subunit	
1013608	G	A	Val50Met	01044	CHP, putative transcriptional regulator	
1653482	G	A	Silent	01748	tRNA-guanine transglycosylase	x
2243145	G	–		R0005	rRNA-16S Ribosomal RNA	
2243146	G	–		R0005	rRNA-16S Ribosomal RNA	
2318272	G	A		Intergenic 002512/02515	Non-coding	x
2318274	G	T		Intergenic 002512/02515	Non-coding	x
2318290	C	A		Intergenic 002512/02515	Non-coding	x
2331612	C	A	Ala30Ser	02527	Peptidoglycan pentaglycine interpeptide biosynthetic protein FmhB (FemX)	
2383630	G	T	Silent	02591	CHP, putative membrane protein	x
2383660	G	T	Silent	02591	CHP, putative membrane protein	x
2466536	T	C	Lys349Glu	02681	Nitrate reductase, alpha subunit	
2556234	A	G		Intergenic 02781/02782	Non-coding	
2596878	C	T	Gly107Asp	02818	MFS family major facilitator transporter	
2678563	T	C	Silent	02911	CHP	x
2684051	C	T	Ala90Thr	02919	3-methyl-2-oxobutanoate hydroxymethyltransferase	
2689048	G	T	Val353Leu	02923	Amino acid permease	x

[1]Genome positions and locus numbers are according to NCTC8325 sequence (GenBank accession CP000253). [2]Reference nucleotide in NCTC8325. [3]Sequenced nucleotide in BB255, RA120 and ME51 determined in this study. [4]Description of putative gene products and functions were taken from NCTC8325 annotations and were improved from annotations of other *S. aureus* strains. [5]x in this column indicates SNP/DIP is present in RN4220 [56]. Abbreviation: CHP, conserved hypothetical protein.
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deletion when assembled against the published SCC*mec* sequence of COL (GenBank accession CP000046). One mutation was found in a glycerophosphoryl diester phosphodiesterase (COL genome position G38611A, SACOL_0031, Asp95Asn) and one in a conserved hypothetical protein (C51476T, SACOL_0044, Cys42Tyr). In the gene of the methicillin resistance surface protein Pls two silent SNPs (G57595A, A61354T) and a five nucleotide deletion at position 61363-61367, leading to the loss of amino acids and a frame shift resulting in truncation of Pls at amino acid 1383, were detected.

In addition to the SNPs and DIPs present in both strains, the genome sequences of RA120 and ME51 differed by only one nucleotide in the recently discovered c-di-AMP cyclase gene *dacA* (SAOUHSC_02407) [29,33], which was confirmed by Sanger sequencing. The SNP was identified in ME51, resulting in the change of a highly conserved glycine to a serine (Gly206Ser,

genome position C2235346T in NCTC8325) which is separated by only one amino acid from the proposed functional motive RHR of the diadenylate cyclase [33].

Reconstruction of the *dacA*-SNP

To confirm a direct connection between the mutation in the *dacA* gene and the phenotypic changes in ME51 that resulted in it becoming a fitter but heterogeneously resistant strain, the mutation was artificially introduced into the homogeneously high-level resistant but slow growing strain RA120 by homologous recombination, resulting in strain RA120::*dacA*-SNP. Upon introduction of the SNP into RA120, its resistance profile became heterogeneous (Figure 1A) and the oxacillin MIC decreased from >256 mg/L for RA120 to 64 mg/L for RA120::*dacA*-SNP. Like ME51, strain RA120::*dacA*-SNP grew faster than RA120 (Figure 1B). The growth rate increased significantly ($p<0.01$) upon introduction of the *dacA*-SNP with a reduction of the doubling time from 39.5±1.7 min for RA120 to 29. 8±1.6 min for RA120::*dacA*-SNP in the exponential growth phase. These resistance and fitness features of RA120::*dacA*-SNP were comparable to ME51 which had a doubling time of 29.0±2.1 and an oxacillin MIC of 64 mg/L. Several recently published studies found that mutations in the c-di-AMP phosphodiesterase *gdpP* increased resistance to beta-lactam antibiotics [38,39,40,41]. The study of Corrigan *et al.* quantified the c-di-AMP levels in a *gdpP* mutant confirming thereby that elevated c-di-AMP levels are associated with increased beta-lactam resistance [29]. We hypothesized that the opposite occurred in ME51. The amino acid substitution close to one of the functional motifs in DacA could reduce the efficiency of the cyclase resulting in a lower c-di-AMP level and decreased beta-lactam resistance. The growth rate of the constructed mutant RA120::*dacA*-SNP increased to a level that was comparable to that of the naturally selected mutant ME51 (Figure 1B), suggesting a direct connection between increased fitness and the mutation in *dacA*.

Quantification of c-di-AMP and penicillin binding proteins (PBPs)

To prove our hypothesis that the *dacA* mutation reduces the cellular level of c-di-AMP, LC-MS analysis of RA120, ME51 and RA120::*dacA*-SNP were performed to quantify c-di-AMP. The amount of c-di-AMP per mg cellular dry weight (cdw) was 13.8±1.7 ng/mg cdw for the wild type strain RA120 and significantly reduced to 5.8±0.2 ng/mg cdw and 4.5±0.5 ng/mg cdw for ME51 and RA120::*dacA*-SNP, respectively (Figure 2A). Compared to the up to 15-fold increase seen in c-di-AMP upon *gdpP* mutation [29] this is a rather small decrease which, however, had a pronounced effect on fitness and resistance.

Pozzi and co-workers showed that a heterogeneously resistant strain was transformed into a homogeneously resistant strain that produced increased amounts of PBP2a, upon mutation in the c-di-AMP phosphodiesterase *gdpP* [41]. Thus, one explanation for a reduction of resistance in the strains ME51 and RA120::*dacA*-SNP could be a decreased level of PBP2a. However, in our strains no significant difference in PBP2a production was detected upon *dacA* mutation neither in ME51 nor in RA120::*dacA*-SNP (Figure 2B). Quantification of signal intensities from PBP2a Western blots gave values of 96±6% for ME51 at OD1 and 104±5% at OD 4, relative to the signal intensity of RA120. Similar values were detected for RA120::*dacA*-SNP resulting in intensity percentages of 98±4% at OD 1 and 106±6% at OD 4, compared to RA120. In the study of Pozzi *et al.* the effect of their *gdpP* mutation on the c-di-AMP level was not quantified, but it may be assumed from the studies of Corrigan *et al.* that *gdpP* mutations have a more drastic

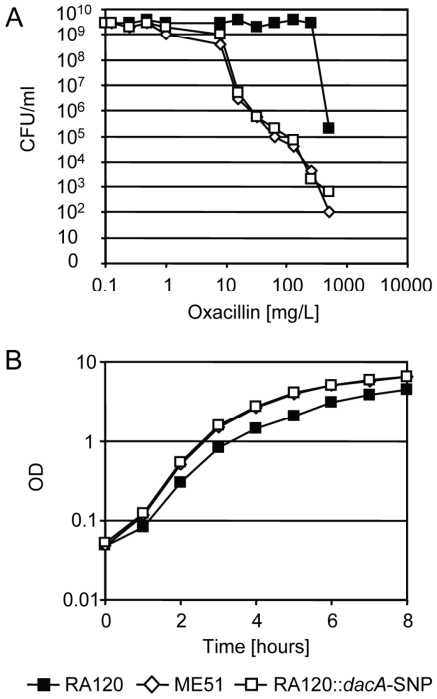

Figure 1. Oxacillin resistance and fitness of RA120, ME51 and RA120::*dacA*-SNP. A, oxacillin population analysis profiles and B, growth curves of the strain RA120 (black squares) and the *dacA*-SNP strains ME51 (white diamonds) and RA120::*dacA*-SNP (white squares). Representative data from three independent experiments are shown.
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effect on c-di-AMP levels than the *dacA*-SNP identified in this study. This could be reflected in the magnitude of PBP2a level changes [29], and explain why we could not detect a difference in PBP2a levels in our *dacA*-SNP mutants. Alternatively, the *dacA*-SNP may influence methicillin resistance independently of the amount of PBP2a. c-di-AMP was previously found to affect cell wall homeostasis and elevated levels of c-di-AMP increased peptidoglycan cross linking by an as yet unknown mechanism [29,40]. These findings, together with the fact that *gdpP* mutations in MSSA strain backgrounds lead to increased beta-lactam MICs [39] and our observation of decreased oxacillin resistance in MSSA strain BB255 upon *dacA*-SNP introduction (see below), suggest that the effects of c-di-AMP on beta-lactam resistance are likely to be at least partially independent of PBP2a levels. However, more research is required to understand the mechanism by which c-di-AMP affects cell wall homeostasis and beta-lactam resistance.

Since PBP2 and PBP4 were previously shown to affect beta-lactam resistance, the protein levels of endogenous PBPs were also analysed [15,16,57,58]. Qualitative analysis of the amount of the endogenous PBPs PBP1, PBP2 and PBP3 using the fluorescent penicillin analogue Bocillin-FL did not reveal any obvious differences between the strains RA120, ME51 and RA120::*dacA*-SNP (Figure 2C). Interestingly, PBP4 Western blots showed seemingly higher amounts of PBP4 in *dacA*-SNP strains ME51 and RA120::*dacA*-SNP, which, however, was not significantly different from RA120 due to large variations between experiments (Figure 2D). Quantification of Western blot signals as in percentages of the

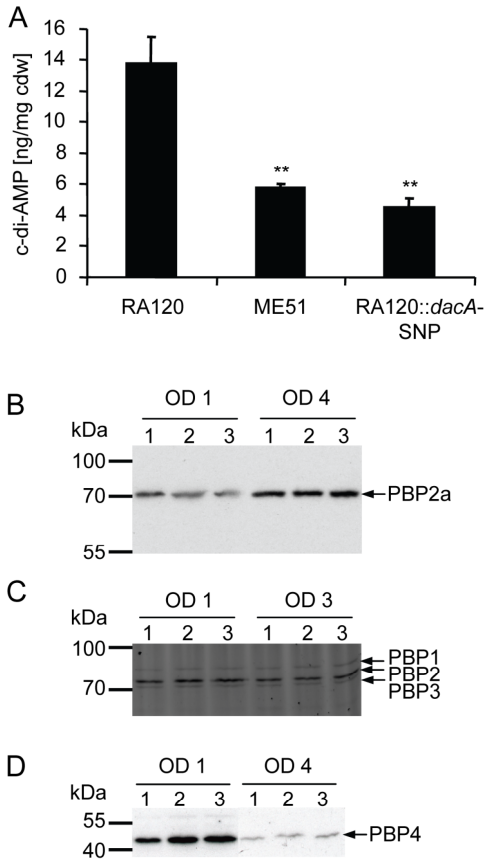

Figure 2. Cellular levels of c-di-AMP and PBPs of RA120, ME51 and RA120::*dacA*-SNP. A, cellular levels of c-di-AMP quantified by LC-MS in ng per mg cellular dry weight (cdw). Average values from five biological replicates with standard deviation are shown. Asterisks indicate significant differences compared to RA120 (** $p<0.01$). B, PBP2a (76 kDa) Western blot analysis, C, visualisation of PBP1 (83 kDa), PBP2 (80 kDa) and PBP3 (77 kDa) using the fluorescent penicillin analogue Bocillin-FL and D, PBP4 (48 kDa) Western blot analysis of membrane fractions of the strains RA120 (1), ME51 (2) and RA120::*dacA*-SNP (3) sampled at indicated ODs. Representative results from three independent protein extractions are shown.
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signal intensity of RA120 gave values of 197±61% and 181±62% at OD 1, and 171±52% and 190±58% at OD 4, respectively. This observation was unexpected, since previous studies had shown that PBP4 levels directly correlated with beta-lactam resistance; overexpression of PBP4 leading to increased beta-lactam resistance [57] and deletion of PBP4 reducing resistance levels [16,58,59]. Even though the importance of PBP4 for beta-lactam resistance was shown to be very strain dependent [16], we would not have expected to see higher PBP4 levels in the *dacA*-SNP strains that had reduced oxacillin MICs. However, it is possible that despite increased total amounts, PBP4 activity or localisation could be impaired and further research is required to evaluate if there is a connection between c-di-AMP levels and PBP4 levels, activity or localisation.

Cell envelope associated phenotypes of *dacA*-SNP

Additional effects of this more than 2-fold decrease in the cellular c-di-AMP level were further investigated by phenotypic characterisation of RA120 and the *dacA*-SNP strains ME51 and RA120::*dacA*-SNP. In *S. aureus*, elevated c-di-AMP levels caused by *gdpP* mutations were found to affect cell envelope homeostasis resulting in increased autolysis and increased resistance to lysostaphin and oxacillin [29]. As expected, we observed the opposite effect in *dacA*-SNP strains. The *dacA*-SNP clearly decreased resistance to oxacillin as shown above (Figure 1A) and decreased both spontaneous and Triton X-100 induced autolysis (Figure 3AB). However, resistance to lysostaphin was not affected (data not shown).

A study in *Lactococcus lactis* revealed salt hypersensitivity in a spontaneous *gdpP* mutant [45]. Although, *S. aureus* can tolerate high salt concentrations [60], we could detect decreased salt sensitivity in the mutants with lower c-di-AMP levels (Figure 3C). Inversely, increased salt sensitivity was observed very recently for a *S. aureus gdpP* mutant with 15-fold increased c-di-AMP levels and for a mutant of the newly identified c-di-AMP target *ktrA*, a potassium transporter-gating component [46].

Since previous studies suggest c-di-AMP to be involved in cell envelope homeostasis and cell envelope stress [29,40] we analysed the effect of the *dacA*-SNP on expression of the cell wall stress stimulon (CWSS). Induction of the CWSS, a set of genes controlled by the two component system VraSR, is a good indicator of cell envelope stress caused by disturbance of cell envelope biosynthesis [19,28,61]. CWSS expression is up-regulated by depletion or deletion of cell envelope biosynthesis enzymes or upon exposure to cell wall-targeting antibiotics [19,61,62,63,64,65,66]. Using the well established reporter plasmid p*sas016*$_p$ *luc*+ which contains the promoter of *sas016*, a highly responsive CWSS gene of unknown function, fused to a luciferase gene [65,67], the CWSS expression was measured indirectly in relative light units (RLU). Indeed, we detected a lower basal CWSS expression over growth in the *dacA*-SNP strains ME51 and RA120::*dacA*-SNP (Figure 3D). The expression level in the parent strain RA120 was about 3-fold higher than in the *dacA*-SNP strains. These findings suggest that cell wall stress stimulon expression could correlate directly with cellular c-di-AMP levels.

Effect of the *dacA*-SNP on fitness, resistance and c-di-AMP levels in an MSSA strain and in an MRSA of different genetic background

To determine the general importance of DacA in different strain backgrounds, the *dacA*-SNP was introduced into the MSSA parent strain BB255 and into the MRSA strain COL which both carried *dacA* genes that were 100% identical to *dacA* in RA120. Both resulting mutants showed increased oxacillin susceptibility (Figure 4A). The number of surviving cells decreased in the methicillin sensitive strain BB255::*dacA*-SNP by approximately 1000-fold on plates containing 0.125 mg/L or 0.25 mg/L of oxacillin, which was also reflected by a reduction of the oxacillin MIC from 0.25 mg/L in BB255 to 0.125 mg/L in BB255::*dacA*-SNP. In the COL::*dacA*-SNP mutant a lowered and heterogeneous resistance profile was observed, though the effect was slightly less pronounced than in the BB255 derived MRSA RA120::*dacA*-SNP. The oxacillin MIC for COL decreased from >256 mg/L to 256 mg/L upon introduction of *dacA*-SNP.

The impact of the *dacA*-SNP on fitness varied between the different strains. The MSSA strain BB255 showed a decrease in the growth rate upon the introduction of the *dacA*-SNP (Figure 4B); the doubling time increased from 30±2.1 min for BB255 to

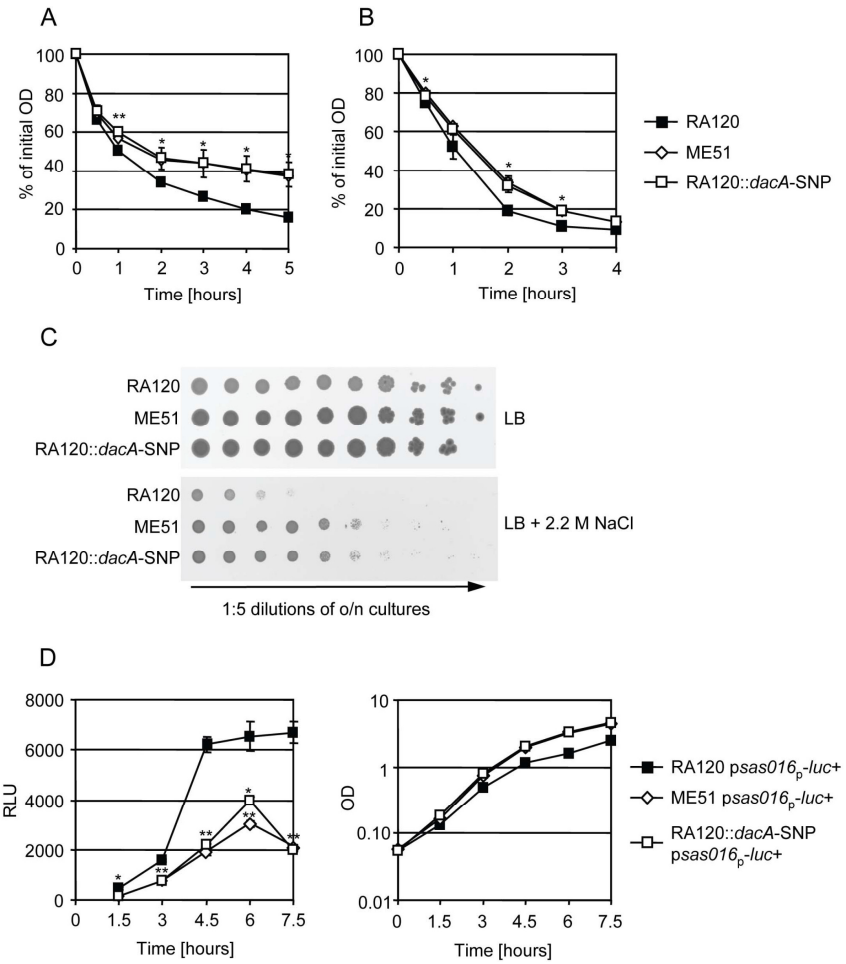

Figure 3. Analysis of phenotypes associated with cell envelope properties in RA120, ME51 and RA120::*dacA*-SNP. A, spontaneous autolysis of RA120 (black squares) and the *dacA*-SNP strains ME51 (white diamonds) and RA120::*dacA*-SNP (white squares) in phosphate buffer and B, induced autolysis of strains with 0.01% Triton X-100 in phosphate buffer. Average values with standard deviation from three independent experiments are shown. Asterisks indicate measurement time points with significant difference between both RA120/ME51 and RA120/RA120::*dacA*-SNP (* $p<0.05$, ** $p<0.01$) using Student's *t*-test. Two-way ANOVA analysis considering all time points resulted in significant differences ($p<0.01$) for stain pairs RA120/ME51 and RA120/RA120::*dacA*-SNP for both spontaneous and induced autolysis. C, NaCl tolerance of RA120, ME51 and RA120::*dacA*-SNP. Overnight cultures were serially diluted 1:5 and plated on plain LB agar or LB agar with 2.2 M NaCl. Representative results from three independent experiments are shown. D, left, relative light units (RLU) measured from cell wall stress stimulon reporter construct p*sas016*$_p$-*luc*+ in RA120 (black squares) and the *dacA*-SNP strains ME51 (white diamonds) and RA120::*dacA*-SNP (white squares) and right, the corresponding OD values of the cultures at each sampling point. Average values with standard deviation from three independent experiments are shown. Asterisks indicate measurement time points with significant differences in RLU between both RA120/ME51 and RA120/RA120::*dacA*-SNP strain pairs. If the strain pairs had different categories of p-values these were indicated separately (* $p<0.05$, ** $p<0.01$). Two-way ANOVA analysis considering RLU values from all time points resulted in significant differences ($p<0.01$) for both strain pairs RA120/ME51 and RA120/RA120::*dacA*-SNP.
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42±3.2 min for BB255::*dacA*-SNP. In COL, the SNP did not significantly affect the minimal doubling time of the strain with 38.2±1.1 min and 40.0±1.9 min for COL and for COL::*dacA*-SNP, respectively (Figure 4C). Quantification of c-di-AMP once again showed clear reductions in cellular c-di-AMP levels upon *dacA*-SNP introduction into both backgrounds (Figure 4D). Introduction of the *dacA*-SNP into MRSA COL reduced the c-di-AMP level about four-fold, from 4.5±0.5 to 1.1±0.1 ng/mg cdw. The induction of *dacA*-SNP into BB255 reduced the c-di-AMP of 2.4±0.3 ng/mg cdw to a level below the limit of detection. The value included in the graph as a grey bar corresponds to the limit of detection (32 nM) divided by samples biomass concentrations. The reduction to a very low c-di-AMP level might be an explanation for the reduced growth rate of BB255::*dacA*-SNP, since c-di-AMP was claimed to be essential for *S. aureus* [29] and reduced c-di-AMP levels were found be detrimental for growth of *B. subtilis* [34] and *L. monocytogenes* [42].

Surprisingly, the c-di-AMP level in BB255, the RA120 parent strain, was about 6-fold lower than the c-di-AMP level in RA120. Therefore, it could be speculated that introduction of the SCC*mec* element into BB255 led to non genetic adaptations of yet unknown nature, resulting in an elevated c-di-AMP level in RA120, which

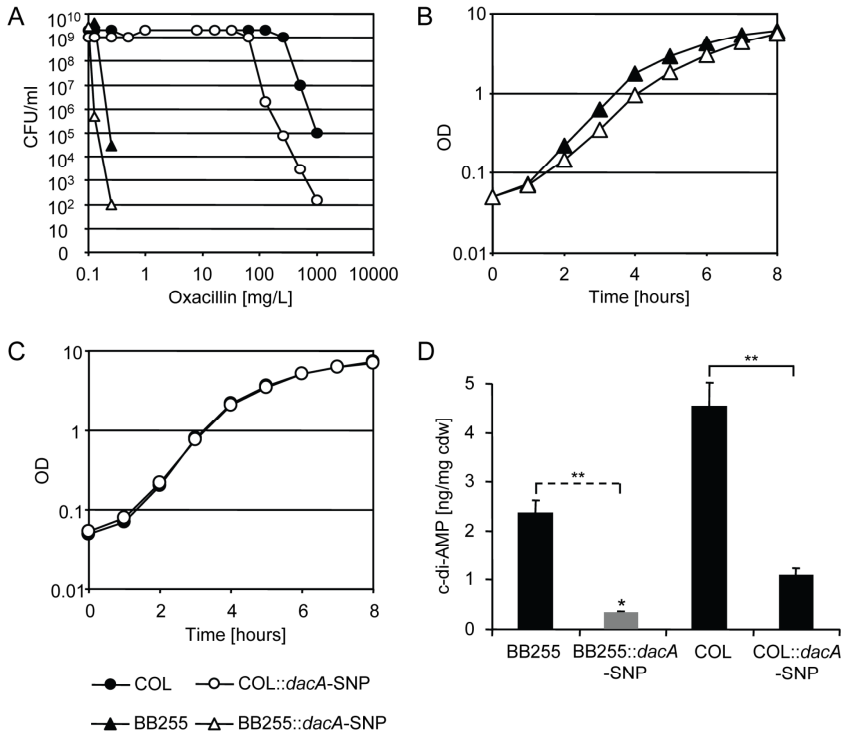

Figure 4. Effect of *dacA*-SNP on oxacillin resistance, fitness and the c-di-AMP levels in MSSA strain BB255 and MRSA strain COL. A, oxacillin population analysis profile of wild type strains BB255 (black triangle) and COL (black circle) and their corresponding *dacA*-SNP strains BB255::*dacA*-SNP (white triangle) and COL::*dacA*-SNP (white circle). Representative data from three independent experiments are shown. B, growth curves of BB255 and BB255::*dacA*-SNP and C, growth curves of COL and COL::*dacA*-SNP. Representative data from three independent experiments are shown. D, cellular levels of c-di-AMP quantified by LC-MS in ng per mg cellular dry weight (cdw). Average values from five biological replicates with standard deviation are shown. Grey bar with asterisk (*) indicates c-di-AMP concentration was blow detection limit and the given value represents maximal concentration which was estimated by dividing the limit of detection (32 nM) by the samples biomass concentrations. Two asterisks (**) indicate a significant difference using Student's *t*-test ($p<0.01$) and the dashed bracket indicates statistical analysis was performed using the estimated maximal value for BB255::*dacA*-SNP.
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was reduced by the *dacA* mutation to a wild type level in ME51 and RA120::*dacA*-SNP. However, to our knowledge, no studies on the natural variation of the c-di-AMP levels in different strain backgrounds have been performed yet and current data suggests that c-di-AMP levels are not generally higher in MRSA, since Corrigan *et al.* did not detect a difference in c-di-AMP levels between MRSA and MSSA [29]. Thus, more work is required to confirm and explain the possibly elevated c-di-AMP levels in RA120 and to generally understand the mechanisms by which c-di-AMP affects beta-lactam resistance. So far, forces driving acquisition of mutations in c-di-AMP synthesising gene *dacA* or the degrading enzyme *gdpP* seem to include stress caused by i) selective pressure by beta-lactams [38,39,41] ii) disturbance of cell envelope integrity due to LTA-deficiency [29] or iii) selective pressure on fitness such as the conditions occurring in mixed growth competition experiments ([54], this work). Why and how altering c-di-AMP levels under these conditions might be an advantage for *S. aureus* remains to be elucidated. As a second messenger, c-di-AMP can be expected to control several factors involved in different cellular processes, which might explain why alterations in c-di-AMP levels can lead to such diverse phenotypes as changes in antibiotic resistance, cell envelope stability, cell division, biofilm formation, growth rate, transcription of regulators, expression of virulence factors and pathogenicity.

Conclusion

This is the first time that a mutation in *dacA*, resulting in decreased c-di-AMP levels, was shown to reduce methicillin resistance and increase growth rates. Markerless allelic replacement confirmed that the *dac*A mutation could transform a highly homogenously methicillin resistant strain into a faster growing but lower-level and heterogeneously resistant MRSA. The impact of the *dacA*-SNP on c-di-AMP levels and fitness seemed to vary between strain backgrounds. Analysis of cell envelope properties of the strains with the *dacA* mutation revealed reduced autolysis, increased salt tolerance and a reduction in the basal expression level of the cell wall stress stimulon, providing further confirmation for a cell envelope associated signalling function of c-di-AMP. Further research is required to understand how cellular c-di-AMP levels influence fitness and resistance. This will involve investigating possible connections between the phenotypes associated with altered c-di-AMP levels and the five recently identified c-di-AMP targets and the potential identification of additional targets of c-di-AMP-dependent regulation.

Supporting Information

Table S1 Complete list of differences in BB255, ME51 and RA120 compared to the NCTC8325 sequence including SNPs and DIPs identified by Berscheid et al. 2012 [55].
(DOC)

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Author Contributions

Conceived and designed the experiments: VD NM PK PC AP JAV BB MMS. Performed the experiments: VD NM PK PC AP MMS. Analyzed the data: VD NM PK PC AP JAV BB MMS. Wrote the paper: VD NM PK JAV BB MMS.

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Table S1: Complete list of differences in BB255, ME51 and RA120 compared to the NCTC8325 sequence including SNPs and DIPs identified by Berscheid *et al.* 2012 [55].

Position[1]	Ref[2]	Seq[3]	Impact	Locus (SAOUHSC_)[1]	Description[4]
5286	G	A	Gly85Ser	00166	DNA gyrase, ATP hydrolyzing subunite B
22181	C	A		Intergenic 00018/00019	Non-coding
47652	T	-		Intergenic 00044/00045	Non-coding
73564	G	T	Asn472Lys	00069	Partial *spa* gene for immunglobulin G binding protein A
142255	G	T	Gly200Trp	00136	CHP, nitrate transport ATP-binding protein NrtD, putative ABC transporter
210632	T	A	Asp454Glu	00190	CHP, membrane domain of membrane-anchored glycerophosphoryl diester phosphodiesterase
218932	C	T	Asp63Asn	00197	Putative acyl-CoA dehydrogenase domain protein
230630	T	G	Silent	00209	Putative PTS system maltose-and glucose-specific EIICB component
252494	G	A	Gly323Asp	00230	Two-component sensor histidine kinase LytS
329229	T	C	Leu131Ser	00314	Possible transcriptional regulator MarR family, MATE family multi-antimicrobial extrusion protein
433209	A	T		Intergenic 00434/00435	Non-coding
523591	G	A	Glu431Lys	00524	DNA-directed RNA polymerase beta subunit
541723	T	G		Intergenic 00535/00536	Non-coding
541724	G	C		Intergenic 00535/00536	Non-coding
590402	G	–	Frame shift	00591	CHP
649126	G	T	Silent	00661	CHP, putative lipase/esterase
841103	G	T	Silent	00877	Iron-sulphur cluster assembly accessory protein
841139	G	T	Silent	00877	Iron-sulphur cluster assembly accessory protein
947898	C	–	Frame shift	00973	Putative glycosyl transferase
980692	C	T	Pro301Leu	01009	Phosphoribosylaminoimidazole carboxylase, ATPase subunit
1013608	G	A	Val50Met	01044	CHP, putative transcriptional regulator

Position[1]	Ref[2]	Seq[3]	Impact	Locus (SAOUHSC_)[1]	Description[4]
1042000	T	–	Frame shift	01078	50S ribosomal protein L32, *rpmF*
1160513	G	A	Ala106Thr	01209	16S rRNA processing protein RimM
1160531	A	G	Lys112Glu	01209	16S rRNA processing protein RimM
1180886	G	–	Frame shift	01232	30S ribosomal protein S2, *rpsB*
1283784	C	–	Frame shift	01342	Exonuclease SbcC
1562913	A	T	Stop337Lys	01649	Peptidase, rhomboid family protein
1632635	–	A	Frame shift	01726	Putative tRNA methyltransferase MnmA
1636251	T	–	Frame shift	01732	BadM/Rrf2 family transcriptional regulator
1653482	G	A	Silent	01748	tRNA-guanine transglycosylase
1683491	T	C	Lys40Glu	01786	Translation initiation factor IF-3 (InfC)
1733515	G	T	Thr73Asn	01827	Septation ring formation regulator EzrA
1733572	A	G	Phe54Ser	01827	Septation ring formation regulator EzrA
1981053	A	G	Phe92Ser	02107	Putative UDP-N-acetylmuramyl-tripeptide synthetase
2087725	A	T	Phe218Ile	02254	Chaperonin GroEL
2166163	G	C	Thr124Arg	02337	UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurA
2166183	C	A	Silent	02337	UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurA
2221850	C	A	Ser244Tyr	02401	Putative transcriptional anti-terminator
2243145	G	–		R0005	rRNA-16S ribosomal RNA
2243146	G	–		R0005	rRNA-16S ribosomal RNA
2244932	C	–	Frame shift	02417	Putative ATP-binding protein, Mrp/Nbp35 family
2244933	T	–	Frame shift	02417	Putative ATP-binding protein, Mrp/Nbp35 family
2296654	G	–	Frame shift	02474	CHP
2318272	G	A		Intergenic 002512/02515	Non-coding
2318274	G	T		Intergenic 002512/02515	Non-coding
2318290	C	A		Intergenic 002512/02515	Non-coding
2331612	C	A	Ala30Ser	02527	Peptidoglycan pentaglycine interpeptide biosynthetic protein

Position[1]	Ref[2]	Seq[3]	Impact	Locus (SAOUHSC_)[1]	Description[4]
					FmhB (FemX)
2349916	G	T		Intergenic 02555/02556	Non-coding
2349964	G	–		Intergenic 02555/02556	Non-coding
2349972	A	–		Intergenic 02555/02556	Non-coding
2349980	T	–		Intergenic 02555/02556	Non-coding
2349986	A	–		Intergenic 02555/02556	Non-coding
2349990	A	–		Intergenic 02555/02556	Non-coding
2349995	T	–		Intergenic 02555/02556	Non-coding
2350002	T	–		Intergenic 02555/02556	Non-coding
2350004	C	–		Intergenic 02555/02556	Non-coding
2350008	A	–		Intergenic 02555/02556	Non-coding
2350012	N	–		Intergenic 02555/02556	Non-coding
2350016	A	–		Intergenic 02555/02556	Non-coding
2350100	T	C		Intergenic 02555/02556	Non-coding
2383630	G	T	Silent	02591	CHP, putative membrane protein
2383660	G	T	Silent	02591	CHP, putative membrane protein
2420619	–	T	Frame shift	02632	Teicoplanin resistance-associated membrane protein TcaB
2446161	C	G	Glu71Asn	02662	PTS system, sucrose-specific IIBC component ScrA
2446162	C	A	Gly70Val	02662	PTS system, sucrose-specific IIBC component ScrA
2446164	C	–	Frame shift	02662	PTS system, sucrose-specific IIBC component ScrA (02661 + 02662 = 1 orf)
2446246	C	T	Silent	02662	PTS system, sucrose-specific IIBC component ScrA
2446393	C	A		Intergenic 02662/02663	Non-coding
2446402	C	–		Intergenic 02662/02663	Non-coding

Position[1]	Ref[2]	Seq[3]	Impact	Locus (SAOUHSC_)[1]	Description[4]
2446423	C	A		Intergenic 02662/02663	Non-coding
2446630	C	T	His26Tyr	02663	CHP
2446641	T	A	Silent	02663	CHP
2466536	T	C	Lys349Glu	02681	Nitrate reductase, alpha subunit
2556234	A	G		Intergenic 02781/02782	Non-coding
2592013	A	–	Frame shift	02813	Hypothetical membrane protein (02813 + 02814 = 1 orf)
2596878	C	T	Gly107Asp	02818	MFS family major facilitator transporter
2678563	T	C	Silent	02911	CHP
2684051	C	T	Ala90Thr	02919	3-methyl-2-oxobutanoate hydroxymethyltransferase
2689048	G	T	Val353Leu	02923	Amino acid permease
2782821	C	–	Frame shift	03008	Imidazole glycerol phosphate synthase subunit HisF

[1]Genome positions and locus numbers are according to NCTC8325 sequence (GenBank accession CP000253). [2]Reference nucleotide in NCTC8325. [3]Sequenced nucleotide in BB255, RA120 and ME51 determined in this study. [4]Description of putative gene products and functions were taken from NCTC8325 annotations and were improved from annotations of other *S. aureus* strains. Abbreviation: CHP, conserved hypothetical protein.

3. 4 Project IV:

Manuscript draft

Characterization of *Staphylococcus aureus* Isolated from Healthy Volunteers Receiving Amoxicillin and Minocycline

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ABSTRACT

Background The opportunistic pathogen *Staphylococcus aureus* remains a major cause for hospital- and community-acquired infections, but is often carried in the nares of the healthy population without causing harm. Because *S. aureus* easily develop resistance or acquire resistance determinants, the effects of minocycline and amoxicillin administration on colonizing *S. aureus* of healthy volunteers was monitored during a 12-month period as part of the FP7 EU-project ANTIRESDEV. **Results** The prevalence of *S. aureus* in the participating volunteers was 47.7 %. All isolates were typed, resistance profiles were determined and growth rates measured as an indication for fitness. The majority of the strains were amoxicillin resistant due to the presence of beta-lactamase. No methicillin-resistant *S. aureus* (MRSA) strains were isolated. Resistance and fitness levels of persisting strains varied over time independent of minocycline or amoxicillin application. No antibiotic resistance development or acquisition of resistance was observed. Volunteers were found to carry just one specific *S. aureus* strain, with few exceptions carrying several strain types. One Volunteer carried two different *S. aureus* strains before amoxicillin administration. After one week amoxicillin treatment, these strains had been replaced by a third strain, which colonized this volunteer during the following two months. All three strains carried a beta-lactamase and were resistant to amoxicillin. However, they differed in the beta-lactamase induction in response to amoxicillin as determined by measuring hydrolysis of the beta-lactamase substrate chromacef. The *S. aureus* strain isolated after amoxicillin administration produced higher beta-lactamase levels upon amoxicillin induction than the first two strains. **Conclusions** The data of this study suggest that the correct application of amoxicillin and minocycline had no measurable effect on the colonizing *S. aureus* isolates in healthy volunteers regarding antibiotic

resistance development or acquisition. Strain specific, varying beta-lactamase induction levels might have led to a replacement of strains in one volunteer upon treated with amoxicillin.

KEYWORDS

ANTIRESDEV, amoxicillin, minocycline, *Staphylococcus aureus*, resistance, beta-lactamase

BACKGROUND

The opportunistic pathogen *S. aureus* can colonize the healthy population persistently or transiently (reviewed in (39)). Carriage of *S. aureus* increases the risk of infections (reviewed in (17)), which can be difficult to treat due to the ability of *S. aureus* to acquire and develop resistances towards virtually all currently used antibiotics (38). Often, resistances inflict a fitness cost because the determinants interfere with important biological processes (1, 8, 15). However, bacteria found ways of reducing the biological costs of resistance, e.g. by tightly regulating the expression of resistance determinants, by reducing the copy number of the elements carrying them, or by combining them with other features adapting their metabolism. One of the best studied resistance determinant of *S. aureus* is the *bla* operon conferring resistance to beta-lactamase sensitive beta-lactams. In the current model, binding of beta-lactams to the sensor transducer BlaR1 leads to autocatalytic cleavage of BlaR1 (40). Thereby BlaR1 is proposed to be activated and to cleave the repressor BlaI (23), leading to the de-repression of the entire *bla* operon encoding BlaR1, BlaI and the beta-lactamase BlaZ (5). Induction is afterwards down-regulated due to the degradation of beta-lactams by BlaZ, eliminating the activating signal, and by shedding of the sensor domain of BlaR1 which reduces the number of responsive BlaR1 proteins and degradation of BlaI (24).

In addition to the ability to acquire a vast range of resistance determinants, the success of *S. aureus* is based on a multitude of virulence factors including microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), secreted expandable repertoire adhesive molecules (SERAMs), proteases, lipases, capsule proteins and toxins. These allow an efficient adhesion to host matrix proteins, evasion of the host innate immune response and invasion of the human body (reviewed in (6)). Different *S. aureus* strains harbor a selection and different combinations of representatives from these virulence factor classes (22, 25). The success of *S. aureus* to colonize the nares is also influence by host factors such as variations between humans in the extracellular matrix produced and in the expression of factors of the innate immune system (reviewed in (17)).

To study the effect of antibiotic administration on the human microbiota, in the FP7 EU-project ANTIRESDEV (http://www.ucl.ac.uk/antiresdev) healthy volunteers were treated either with amoxicillin, minocycline, clindamycin, ciprofloxacin or placebo and samples from several body sites were collected during a 12-month period (2). Here we present data for *S. aureus* isolates from the amoxicillin and minocycline study. A subset of strains was further characterized regarding the

expression of beta-lactamase, autolytic behavior, adhesion to and survival in the presence of lung epithelial cells and biofilm formation.

RESULTS AND DISCUSSION

Characterization of *S. aureus* isolates

Of the 44 volunteers participating in the minocycline (MIN) and amoxicillin (AMX) ANTIRESDEV study (2), twenty-one (47.7 %) were carrying *S. aureus* transiently or persistently (Table 1). *S. aureus* was isolated mainly from the nose (86 of 90 isolates, 95.56 %) and four isolates (0.04 %) were sampled from the skin of the trunk (Tables 2-4). ANTIRESDEV volunteers were recruited in the nearer area of London, UK (2). Compared to a recent survey reporting a 25.4 % *S. aureus* prevalence in the UK (7), the percentage of our study was rather high. In the study of den Heijer *et al*. over 3000 patients with non-infectious disorders visiting family doctors both in rural and urban areas were screened for *S. aureus* (7), thus different recruitment strategies and volunteer numbers might explain the differences in prevalence found. To determine the relatedness of the *S. aureus* strains, all isolates were typed by pulsed-field gel electrophoresis (PFGE). One representative strain per PFGE pattern was further characterized by multi-locus sequence typing (MLST), as well as *spa*, *agr* and *ccr* typing (Tables 2-4). Volunteers generally carried one specific strain, with the exception of volunteers 21 (V021), 24 (V024) and 30 (V030). In V021, two different strains were isolated before the administration of AMX, ARD147 (isolate N-021-2010-9-29/A1) and ARD148 (isolate N-021-2010-9-29/A2). After administration of the antibiotic, these had disappeared and instead a third strain was isolated ARD152 (isolate N-021-2010-10-6/A1) (Table 4). V024 was transiently colonized by two different strains after one month (N-024-2010-8-18/A1) and after 12 months (N-024-2011-7-20/A1) of AMX administration (Table 4). Also V030 was only transiently colonized with *S. aureus*: two different strains were found in the original sample N-030-2010-11-24/A3 collected after one month of AMX administration, of which only one (N-030-2010-12-20/A1) was isolated after two months (Table 4) .

Some of the volunteers were colonized discontinuously with their *S. aureus* strain, which could be due to a recovery rate of *S. aureus* below 100 % by nasal swabbing, as observed by others (28). Alternatively, transiently colonized nares from these volunteers could have been re-colonized from an endogenous source, i.e. from other body sites that might have been persistently colonized by their specific *S. aureus* strain.

A total of 26 *S. aureus* strains with different PFGE types were MLST typed resulting in 12 different sequence types (ST) in the volunteers colonized with *S. aureus*. The most frequently found sequence types were ST5 (7 or 26.9 %), ST30 (4 or 15.3 %), ST8, ST45 and ST54 (all 2 or 7.6 %). Similar results were found in other studies (28). Two new sequence alleles were identified and deposited in the MLST database. For *spa* types, t002 was most frequently detected with 4 isolates (15.3 %), followed

by t008, t012 and t015 with each 2 isolates (7.6 %); these types rank among the top 11 in the current Ridom database, collecting *spa* types from all over the world (http://spa.ridom.de/index.shtml). In the isolates analyzed, we found *agr* types 1-3, but no *agr* 4 type. Despite the fact that no MRSA strains were obtained, we identified in the isolates from two volunteers, V030 and V037, *ccr* genes of the types 2 and 1, respectively. The *ccr* recombinases are typically found on staphylococcal cassette chromosome (SCC) elements harboring *mecA*, the gene encoding the beta-lactam resistance determinant PBP2a (14). However, *ccr* genes can also be associated with non-*mecA* SCC elements harboring other resistance determinants and virulence factors (27).

To identify antibiotic resistances arising or present in the *S. aureus* strains, antibiograms were determined for a broad range of antibiotics and minimal inhibitory concentrations (MICs) for amoxicillin, minocycline, clindamycin, ciprofloxacin and erythromycin were assessed. Minimal doubling times under standard laboratory conditions were measured as an indication for fitness (Tables 2-4). Both MICs and doubling times varied over the time monitored, suggesting that strains were adapting to shifting conditions in the human host. However, no resistance development or acquisition was observed. Standard deviations of doubling times were rather large and no significant differences could be found during the time monitored. In addition, the number of volunteers being colonized with *S. aureus* at least over four consecutive samplings was limited to four, making it difficult to produce enough data for testing a possible link between changes in MIC levels to changes in doubling times.

Stress tolerance of isolates from volunteer V021

The exceptional colonization of volunteer V021 with three different *S. aureus* strains (ARD147, ARD148 and ARD152) within a relatively short time called for a more in-depth characterization of these isolates. Moreover, strains ARD147 and ARD148 were sampled before the treatment of volunteer V021 with AMX and disappeared thereafter, whereas strain ARD152 appeared after one week of antibiotic administration. Thus, it seemed possible that ARD152 had competitively replaced the strains ARD147 and ARD148. The three strain types were therefore analyzed regarding their ability to grow on different media, as an indication of their flexibility and ability to adapt to stressful conditions they might encounter in the nares or on the skin: desiccation/re-hydration, osmotic stress due to high salt concentration, low pH and fatty acids like linoleic acid (3, 16, 36).

Desiccation and re-hydration tolerance was tested by determining the reduction of viability of V021 isolates after a desiccation period of 7 days (Figure 1). No difference in the extent of viability loss due to desiccation was found, suggesting that ARD147 and ARD148 type strains had not disappeared due to a reduced ability to survive dehydration in the human nose.

In addition, different media imposing various stress types typically encountered on the human body as part of the human innate host defense were tested: Growth at low pH, high salt concentration (a stress *S. aureus* undergoes for example in axillae after sweating, a site from where re-colonization of the

nose can happen) and the presence of long chain unsaturated free fatty acids such as linoleic acid, a major component of nasal fluids. All the isolates from volunteer V021 grew similarly well under such conditions (Figure 2).

The production of certain enzymes, such as proteases and lipases, might favor nutrient acquisition. In addition, expression of extracellular carbohydrates, such as capsule or polysaccharide intercellular adhesin (PIA) can protect *S. aureus* from harmful substances like antibiotics or antimicrobial peptides. In addition, PIA is involved in biofilm formation, where cells again are relatively shielded from harmful substances and can persist locally while continuously shedding cells for dissemination in the human body. Congo red plates, where carbohydrate-positive colonies appear dry/crystalline, Müller-Hinton (MH) plates containing Tween 60, a substrate for lipases, and skim milk plates for detection of proteases were used to test the ability of the V021 isolates to grow on diverse media and for the production of extracellular carbohydrates, proteases and lipases (Figure 3). Isolates ARD147 and ARD148 appeared bright red on Congo red plates clearly different from isolates sampled after AMX administration (ARD152, 153, 275-277, 292-294) which were dark and dry suggesting they produce more extracellular carbohydrates. ARD147 and ARD148 seemed to produce more lipases though, as judged from the pronounced halos observed on MH-Tween 60 plates, caused by precipitation of degradation products from stearic acid, the main component of Tween 60. Pigment formation on skim milk plates was much higher in the isolates ARD147 and ARD148 compared to later sampled isolates (ARD152, 153, 275-277, 292-294), which appeared flat and almost transparent by eye but were able to grow as well as ARD147 and ARD148 (Figure 3D). Protease production was almost undetectable in strains ARD148 and ARD152, whereas strain ARD147 was a strong protease producer (Figure 3E). Since the isolates ARD152, 153, 275-277, 292-294 behaved similarly under the tested conditions so far and showed the same PFGE profiles, ARD152 was chosen as a representative strain for the subsequent experiments.

Biofilm formation can be involved in infections of the nose, ear and throat, and was therefore assessed to determine whether persistent colonization of volunteer V021 by strain type ARD152 was due to higher biofilm formation. ARD152 produced slightly more biofilm than strains ARD147 and ARD148 (Figure 4), but the difference was not significant and the impact for persistence might be negligible.

Autolysis of V021 isolates

Cell envelope stability protects bacteria from many harmful substances, including antibiotics, salt stress and human innate defense mechanisms that destabilize the cell membrane, such as antimicrobial peptides or fatty acids (16, 30). Therefore, we tested the stability of the isolates ARD147, ARD148 and ARD152 by performing autolysis assays. Spontaneous autolysis was tested under low ionic conditions, where autolysins are activated due to a dissipation of cations and protons from the envelope. Peptidoglycan, proteins, lipids and wall teichoic acids can influence cation homeostasis of

the outer surroundings of the cell. Autolysis can be induced by adding the detergent Triton X-100 (TX), which has been reported to remove lipoteichoic acids that protect form autolysins.
In the absence of an inducer, autolysis rates of the strains ARD147 and ARD148 were very similar; autolysis of strain ARD152 was slightly increased (Figure 5A). Addition of 0.01 % TX reproducibly reduced autolysis rates of strain ARD147 and ARD148 in the beginning before reaching similar levels as strain ARD152, whose autolysis rate was further increased by the addition of TX (Figure 5B).

Beta-lactamase expression of V021 strains

To further characterize the V021 isolates, we determined the activity of the beta-lactamase enzyme BlaZ under induced and uninduced conditions by measuring the hydrolysis of the substrate chromacef. Strains ARD147 and ARD152 induced their BlaZ in response to AMX stress, whereas in strain ARD148 no Blaz activity was detected (Figure 6). Interestingly, strain ARD152 had higher BlaZ activity than ARD147 both under induced and uninduced conditions.
Since we could not detect beta-lactamase activity in strain ARD148, we performed control PCRs to confirm the presence of the gene *blaZ*. All three strains contained *blaZ* genes, but could only be amplified with certain primer pairs, indicating that their nucleotide sequence varied (data not shown). To assess whether *blaZ* encoded a functional beta-lactamase, disc tests using penicillin G were performed. In agreement with amoxicillin MIC determinations (Table 4), all three V021 isolates were resistant towards penicillin G according to EUCAST guidelines (R < 26 mm) and produced sharp zone edges (data not shown), which indicate the presence of a functional beta-lactamase. However, the inhibition zone diameter of ARD148 was considerably larger than the ones from ARD147 and ARD152 (25 mm, 17 mm and 18 mm, respectively), suggesting that the induction, production, secretion or activity of the beta-lactamase in ARD148 was reduced compared to strains ARD147 and ARD152.

Adhesion, internalization and survival in non-professional phagocytes

For adhesion to host cells, an important step for colonization, *S. aureus* produces a large and variable arsenal of surface proteins which allow binding of the bacterium to the host matrix. Internalization into non-professional phagocytes, such as epithelial cells can follow, and depending on the survival abilities, *S. aureus* can also persist and hide in host cells.
Strains ARD147, ARD148 and ARD152 were tested for adhesion, internalization and survival using the lung epithelial cell line A549 (10). Strains ARD147 and ARD148 adhered better to A549 cells than ARD152 represented by higher percentages of adhered cells after one hour incubation relative to the initial inoculum (Figure 7A). Surprisingly, ARD152 seemed to be more invasive than strains ARD147 and ARD148 after a three hour incubation period. For invasion percentages of invasive bacteria relative to the inoculum incubated in cell culture medium for three hours are given (Figure 7B).

CONCLUSIONS

Treatment of healthy volunteers with amoxicillin and minocycline did not alter resistance or fitness levels of the colonizing *S. aureus* strains. No methicillin resistant strains were found, but the majority of the strains were positive for beta-lactamase and resistant to amoxicillin. No acquisition or development of resistance neither towards the used antibiotics nor to other antibiotics was observed. These data suggest that resistance development and acquisition does not frequently occur during standard antibiotic treatment.

The majority of the volunteers carried just one *S. aureus* strain during the time monitored. The isolates from volunteer V021 were characterized into more detail because of the possibility that the strain ARD152, which was isolated after the amoxicillin treatment, could have competitively replaced the two strains ARD147 and ARD148 initially present in the nares. However, in the majority of the experiments carried out to determine the fitness of the three strains under various conditions, no difference was found between the three strains or ARD152 was found to be the least fit. However, the three strains differed in induction of their beta-lactamase activity in response to amoxicillin determined by measuring hydrolysis of the beta-lactamase substrate chromacef. Strain ARD152 produced higher beta-lactamase levels than strains ARD147 and ARD148 upon induction. Although caution has to be taken by drawing conclusions from the results produced under laboratory conditions, this could have been an advantage for ARD152 during amoxicillin treatment and could have contributed to the disappearance of ARD147 and ARD148, leading to the colonization of volunteer V021 with strain ARD152 during two months.

METHODS

Origin of *S. aureus* isolates.

Detailed information about the ANTIRESDEV minocycline (MIN) and amoxicillin (AMX) study design has been described elsewhere (2). Briefly, healthy volunteers (aged 18-40) without hospital admission or antibiotic treatment during the preceding 3 months were recruited from the nearer London area in the UK. Volunteers received either MIN (100 mg once daily for 7 days) or AMX (250 mg three times per day for 7 days) or placebo. Samples from skin, nose, saliva and faeces were taken before and 7-10 days, 1 month, 2 months, 4 months and 12 months after administration of antibiotics.

Typing of isolates.

S. aureus isolates received from study partner were typed by PFGE as described previously (31). MLST typing was performed according to Enright *et al*. (9), *spa* types were determined following instructions given in (11). Typing of *agr* was done according to Lina *et al*. (21). *ccr* types were determined based on previous reports (12, 13, 19). Primers used are listed in Table 5.

Resistance profiles.

Minimal inhibitory concentrations for MIN, AMX, clindamycin, ciprofloxacin and erythromycin were determined using the agar dilution method according to EUCAST guidelines (http://www.eucast.org). Additionally, antibiotic susceptibility was analysed using disc diffusion assays. Bacterial suspensions were adjusted to McFarland turbidity standard 0.5. Suspensions were swabbed on Müller-Hinton agar, antibiotic discs (SirScan® discs i2a, Montpellier, France) were spotted and plates were incubated at 37°C for 24 h.

Growth and minimal doubling times.

Growth curves were monitored in Luria-Bertani (LB) broth using a plate reader (Power Wave XS, BioTek, Winooski, VT, USA) and minimal doubling times were calculated. Optical density at 600 nm (OD_{600}) was measured every 3 min after short shaking of the plate.

Desiccation experiments.

Based on previous reports (3, 18) desiccation tolerance was tested as follows: Bacteria were grown overnight in LB, adjusted to an OD_{600} of 1. Twenty µl were dispensed into microtiter dishes, and either immediately serially diluted up to 10^{-7} for spotting on LB agar plates or dried in a laminar flow hood. After 7 days, 100 µl LB was added to the wells, plates were incubated for 15 min at room temperature to allow rehydration and bacteria were resuspended by repeated pipetting. Suspensions were diluted and spotted on LB agar plates as described above and compared to the plates from day zero.

Lipase detection.

Bacteria were prepared as described for the desiccation experiment and spotted on Müller-Hinton agar plates containing 0.01 % $CaCl_2$ and 1 % Tween 60 (35-50 % palmitic acid, 47-55 % stearic acid, Sigma, St. Louis, MO, USA). Plates were incubated at 37°C at least three days and up to one week based on recommendations (20).

Growth under stress conditions.

Bacteria were prepared as described above and spotted on LB agar plates adjusted to pH 7 or pH 5, containing 2 M NaCl or increasing amounts of linoleic acid. Plates were incubated at 37°C for 24-48 hours.

Biofilm formation.

Instructions described by Seidl *et al*. (33) were applied as follows: Bacteria were grown overnight in BHI supplemented with 1 % glucose (BHIG). The OD was adjusted to 0.05 with BHIG and 1 ml was added to 12-well flat bottom Nunclon Delta-treated polystyrene plates (Thermo Scientific, Waltham, MA, USA) in triplicate. Plates were incubated for 18 hours at 37°C. The medium was aspired and the adhering bacteria were washed three times with PBS pH 7.4. Plates were dried and cells were stained with a 0.1 % safranin solution. After washing three times with dH_2O, 200 µl of a 30 % acetic acid

solution were added to the wells to dissolve the adherent dye. The absorbance of the suspended dye was measured at 530 nm as an indication for biofilm formation. Medium without bacteria was used as a negative control.

Autolysis.

Experiments were performed basically as previously described (32). Briefly, cells were grown to exponential growth phase (OD_{600} 0.5), washed with 0.85 % NaCl and resuspended in 0.01 M Na-phosphate buffer pH 7. The OD_{600} was adjusted to 0.5. Cultures were split in half and 0.01 % Triton X-100 (Fluka) or an equal volume of PBS pH 7 was added. Cultures were incubated at 37°C and the decrease of OD_{600} was measured.

Beta-lactamase testing.

Overnight cultures were diluted to an OD_{600} 0.05 and grown to an OD_{600} 0.5 at 37°C in a shaking incubator. One ml of culture was snap-frozen in liquid nitrogen (time point zero) and the culture was split in two halves. One half was induced with 0.5 MIC AMX, the other with an equal volume of dH_2O. Cultures were grown for another two hours. Every 20 min OD_{600} was recorded and 1 ml samples were taken and snap-frozen. For measuring beta-lactamase activity, samples were thawed on ice. Three 200 µl aliquots were adjusted to 990 µl with LB and kept on ice until the addition of 10 µl of a 10 mM chromacef solution (Sopharmia Inc., St. Joseph, USA), which structurally resembles the standard beta-lactamase substrate nitrocefin. Samples where then incubated at 37°C for 30-60 min, centrifuged at 16'000 g for 1 min in a table top centrifuge and the supernatant was transferred to a cuvette for measuring the absorption of the chromacef degradation product at 442 nm and at 600 nm to determine the amount of any remaining cellular debris. One ml LB was treated like the samples and used as blank. Beta-lactamase activity is given as degraded chromacef in nmol OD_{600}^{-1} min^{-1}.

Adhesion and internalisation experiments.

Adhesion and internalization experiments were performed as described before (4). Briefly, overnight cultures of *S. aureus* strains were diluted to OD 0.2 in PBS and 300 µl of the cell suspensions, corresponding to approximately 10^5 cfu/ml, were added to 10 ml DMEM plus (DMEM with high glucose, pyruvate, L-glutamine (Invitrogen, Carlsbad, CA, USA); plus 10 % fetal bovine serum (Sigma)). A549 cells were grown to confluency in 12-well cell culture plates, 1 ml *S. aureus* suspensions in DMEM plus were added and plates incubated at 37°C and 5 % CO_2 for 1 hour or 3 hours. Afterwards, cell layers were resolved using 0.25 % trypsin/EDTA (Sigma) after 10 min treatment with 10 mg/l lysostaphin to for internalization experiments (lysostaphin protection assay (35)). cfu of the inoculum and of bacteria recovered from cell cultures were determinate by plating appropriate dilutions in 0.85 % NaCl on sheep blood agar. Four replicates per experiments and three independent experiments in total were performed.

AVAILABILITY OF SUPPORTING DATA

New MLST alleles and STs, as well as new *spa* types were submitted to the corresponding database curators for subsequent addition to the databases.

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

VD determined doubling times, tested antibiotic susceptibilities, identified *agr* types, contributed to PFGE, MLST, *spa* and *ccr* typing, adhesion and internalization experiments; and drafted the manuscript. SH contributed to MLST, *ccr*, and *spa* typing and adhesion and internalization experiments. MD contributed to the determination of doubling times, tested antibiotic susceptibilities and performed PFGE, *spa* and *ccr* typing. CE measured autolysis and beta-lactamase induction. FS analyzed *spa* sequence types. GP helped with *spa* type interpretation and provided intellectual input. VP and JW performed microarray hybridizations for detection of resistance markers and virulence determinants. BBB provided intellectual input and revised the manuscript. NM designed the study, performed PFGE typing and contributed to manuscript writing. MMS contributed to the study concept, performed stress tolerance experiments, determined expression of extracellular virulence factors, tested biofilm formation and finalized the manuscript.

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Table 1. Colonization pattern of volunteers carrying *S. aureus* during the ANTIRESDEV study.

A) Placebo group (14 volunteers).

V	# SA	0	1 week	1 month	2 months	4 months	1 year
011	2						
028	6						
040	4						

B) Minocycline group (15 volunteers).

V	# SA	0	1 week	1 month	2 months	4 months	1 year
003	1						
004	1						
019	1						
022	4						
034	1						
037	6						
042	4						
129	2						

C) Amoxicillin group (15 volunteers).

V	# SA	0	1 week	1 month	2 months	4 months	1 year
006	8						
008	11						
015	9						
017	5						
021	10						
024	2						
027	7						
030	2						
035	1						
139	2						

V, volunteer; # SA, total number of *S. aureus* isolates; yellow, colonized with *S. aureus*; grey, volunteer dropped out of study.

Table 2. Typing results, doubling times and antibiogram of *S. aureus* isolates from the placebo group.

UZH #	Isolate	PFGE	MLST	*spa*	*agr*	Dt [min]	MIC [µg/ml]					Resistance phenotype												
							AMX	CIP	CLI	MIN	ERY	AMC	AMK	AMP	CIP	CLI	ERY	GEN	FOX	LVX	RIF	SXT	TEC	TET	TOB
74	ST011-2010-6-24/A12	11	ST5	t002	2	28.3	**0.5**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
75	ST011-2010-6-24/A13	11				28.5	**1**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
128	N-028-2010-8-4/A2	28A	ST45	t1976	1	28.6	0.125	0.25	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
129	N-028-2010-8-9/A1	28A				28.4	0.125	0.25	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
132	N-028-2010-8-9/A1	28A				27.5	0.125	0.25	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
140	N-028-2010-9-2/A1	28A				28.3	**0.25**	0.25	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
146	N-028-2010-9-27/A1	28A				27.5	0.125	0.5	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
291	N-028-2010-11-29/A1	28A				28.9	**0.25**	0.25	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
272	N-040-2010-10-27/A1	40	ST582	t084	2	27.8	**0.25**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
273	N-040-2010-11-1/A1	40				27.8	**0.25**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
286	N-040-2010-11-22/A8	40				27.9	**0.25**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
296	N-040-2010-12-20/A1	40				30.5	**0.25**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S

Table 3. Typing results, doubling times and antibiogram of *S. aureus* isolates from the minocycline group.

UZH #	Isolate	PFGE	MLST	*spa*	*agr*	Dt [min]	MIC [µg/ml]				Resistance phenotype														
							AMX	CIP	CLI	MIN	ERY	AMC	AMK	AMP	CIP	CLI	ERY	GEN	FOX	LVX	RIF	SXT	TEC	TET	TOB
4	N-003-2010-5-2/2	3A	ST5	t548	2	26.1	**0.25**	0.5	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S	S
135	N-004-2010-9-8/A6	4	new	t216	1	28.7	**2**	1	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
271	N-019-2010-10-27/A1	19	ST45	t031	1	26.3	0.125	1	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S	S
66	N-022-2010-7-14/A1	22	ST7	t091	1	27.8	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
69	N-022-2010-7-19/A1	22				28.1	**2**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
133	N-022-2010-8-13/A1	22				27.4	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
281	N-022-2010-11-8/A1	22				28.3	**2**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
149	N-034-2010-10-6/A1	34	ST1462	t2883	1	28.2	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
138	N-037-2010-8-25/A1	37	ST96	t521	3	31.0	**2**	0.25	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	nd	S	S
139	N-037-2010-8-25/A2	37				28.0	**2**	0.25	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
141	N-037-2010-8-30/A1	37				28.1	**2**	0.25	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
145	N-037-2010-9-20/A1	37				28.0	**2**	0.25	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
269	N-037-2010-10-18/A1	37				27.3	**0.25**	0.25	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
295	N-037-2010-12-15/A1	37				26.9	**1**	0.25	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
274	N-042-2010-11-1/A1	42	new	t2292	1	26.3	**2**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
298	N-042-2010-12-29/A1	42				26.3	**2**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
305	N-042-2011-9-15/A1	42				27.6	**1**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
306	N-042-2011-9-15/A2	42				28.3	**1**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S
288	N-129-2010-11-24/A1	129A	ST34	t6806	3	31.0	**1**	0.25	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S	**R**

Table 4. Typing results, doubling times and antibiogram of *S. aureus* isolates from the amoxicillin group.

UZH #	Isolate	PFGE	MLST	*spa*	*agr*	Dt [min]	MIC [µg/ml]					Resistance phenotype												
							AMX	CIP	CLI	MIN	ERY	AMC	AMK	AMP	CIP	CLI	ERY	GEN	FOX	LVX	RIF	SXT	TEC	TET	TOB
136	N-006-2010-9-15/A1	6	ST30	t037	3	27.7	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
137	N-006-2010-9-15/A3	6				26.9	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
142	N-006-2010-9-8/A1	6				27.9	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
150	N-006-2010-10-6/A1	6				28.4	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
278	N-006-2010-11-3/A1	6				27.6	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
279	N-006-2010-11-3/A3	6				28.2	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
299	N-006-2011-1-5/A1	6				27.6	**1**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
307	N-006-2011-9-7/A1	6				28.9	**0.5**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
9	N-008-2010-5-17/O2 1	8	ST2021	t008	1	30.4	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
10	N-008-2010-5-17/O2 2	8				30.4	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
11	N-008-2010-5-17/O2 3	8				29.6	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
12	N-008-2010-5-24/O2 2	8				30.4	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
13	N-008-2010-5-24/O2 4	8				30.6	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
15	N-008-2010-6-14/O2 1	8				30.4	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
16	N-008-2010-6-14/O2 4	8				30.1	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
67	N-008-2010-7-14/A1	8				29.6	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
68	N-008-2010-7-14/A4	8				30.2	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
143	N-008-2010-9-8/A1	8				30.0	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
144	N-008-2010-9-8/A3	8				29.9	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
14	N-015-2010-6-9/O2 1	15	ST30	t012	3	29.9	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
17	N-015-2010-6-16/O2 1	15				27.7	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
64	N-015-2010-7-7/A2	15				28.7	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
65	N-015-2010-7-7/A4	15				30.4	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
130	N-015-2010-8-11/A1	15				31.4	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
131	N-015-2010-8-11/A7	15				30.7	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
156	N-015-2010-10-6/A1	15				28.7	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
302	N-015-2011-6-7/A4	15				35.4	**0.5**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
303	N-015-2011-6-7/A5	15				31.6	**0.5**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
22y	N-017-2010-6-23/O2 2	17	ST5	t002	2	29.0	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S

Table 4 - continued.

147	N-021-2010-9-29/A1	21A	ST5	t002	2	27.3	**4**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
148	N-021-2010-9-29/A2	21B	ST8	t1476	1	28.0	**2**	**2**	0.125	0.25	0.5	S	S	**R**	S	S	S	S	S	S	S	S	**R**	S
152	N-021-2010-10-6/A1	21C	ST30	t012	3	29.0	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
153	N-021-2010-10-6/A8	21C				28.9	**2**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
275	N-021-2010-10-27/A3	21C				28.8	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
276	N-021-2010-10-27/A6	21C				29.8	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
277	N-021-2010-10-27/A7	21C				29.4	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
292	N-021-2010-12-6/A1	21C				29.1	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
293	N-021-2010-12-6/A9	21C				29.0	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
294	N-021-2010-12-6/A10	21C				29.8	**1**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
134	N-024-2010-8-18/A1	24A	ST8	t008	1	29.6	**1**	0.5	0.125	0.0625	0.5	S	S	S	S	S	S	S	S	S	S	S	S	S
308	N-024-2011-7-20/A1	24B	ST5	t179	2	26.8	**1**	0.5	0.0625	0.125	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
70	N-027-2010-7-28/A3	27	ST30	t021	3	25.5	**0.25**	1	0.125	0.0625	**32**	S	S	**R**	S	S	**R**	S	S	S	S	S	S	S
126	N-027-2010-8-4/A1	27				29.6	**0.25**	1	0.125	0.0625	**32**	S	S	**R**	S	I	**R**	S	S	S	S	S	S	S
127	ST-027-2010-8-4/A1	27				30.4	**0.25**	1	0.125	0.0625	**32**	S	S	**R**	S	I	**R**	S	S	S	S	S	S	S
154	N-027-2010-10-6/A5	27				25.9	**0.25**	1	0.125	0.0625	**32**	S	S	**R**	S	I	**R**	S	S	S	S	S	S	S
155	N-027-2010-10-6/A6	27				27.0	**0.25**	1	0.125	0.0625	**32**	S	S	**R**	S	I	**R**	S	S	S	S	S	S	S
287	N-027-2010-11-22/A2	27				29.1	**0.25**	0.5	0.0625	0.0625	**32**	S	S	**R**	S	I	**R**	S	S	S	S	S	S	S
313	N-027-2011-11-26/A2	nd				27.6	**0.25**	0.5	0.125	0.0625	**32**	S	S	**R**	S	I	**R**	S	S	S	S	S	S	S
289 m	N-030-2010-11-24/A3	30	mix	nd	1	nd	**1**	0.25	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
289_1	N-030-2010-11-24/A3		ST182	t493	1	31.5	**0.25**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
289_3	N-030-2010-11-24/A3		ST54	t015	1	28.9	**1**	0.5	0.125	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
297	N-030-2010-12-20/A1	30	ST54	t015	1	26.6	**1**	0.25	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
280	N-035-2010-11-1/A1	35A	ST5	t002	2	27.0	**0.25**	0.5	0.0625	0.0625	0.5	S	S	**R**	S	S	S	S	S	S	S	S	S	S
300	N-139-2011-3-17/A2	139	ST5	t311	2	26.6	**4**	0.5	0.0625	0.125	**64**	S	S	**R**	S	S	**R**	S	S	S	S	**R**	S	S
301	N-139-2011-3-17/A3	139				25.6	**4**	0.5	0.0625	0.125	**64**	S	S	**R**	S	S	**R**	S	S	S	S	**I**	S	S

Abbreviations and explanations for Tables 2-4:

UZH #	UZH designation of isolates, in the text preceded by “ARD”
PFGE	pulsed field gel electrophoresis type
MLST	multi-locus sequence typing
spa	staphylococcal protein A type
agr	accessory gene regulator type
ccr	cassette chromosome recombinase type
Dt	doubling time
AMC	amoxicillin-clavulanic acid
AMK	amikacin
AMP	ampicillin
AMX	amoxicillin
CIP	ciprofloxacin
CLI	clindamycin
ERY	erythromycin
FOX	cefoxitin
GEN	gentamicin
LVX	levofloxacin
MIN	minocycline
nd	not determined
RIF	rifampicin
SXT	trimethoprim-sulfamethoxazole
TEC	teicoplanin
TET	tetracycline
TOB	tobramycin

EUCAST breakpoints

AMX	S ≤ 0.125 mg/l < R
CIP	S ≤ 1 mg/l < R
CLI	S ≤ 0.25 mg/l, 0.5 mg/l < R
ERY	S ≤ 1 mg/l, 2 mg/l < R
MIN	S ≤ 0.5 mg/l, 1 mg/l < R

Resistant phenotype in bold

Table 5. Oligonucleotides used in this study.

Designation	Sequence (5'-3')	Remarks	Reference
α2	AACCTATATCATCAATCAGTACGT	*ccr1* typing, rev	(12)
α3	TAAAGGCATCAATGCACAAACACT	*ccr2* typing, rev	(12)
α4	AGCTCAAAAGCAAGCAATAGAAT	*ccr3* typing, rev	(12)
α4.2	GTATCAATGCACCAGAACTT	*ccr4* typing, fwd	(19)
*agr*1-4$_{Sa}$-1	ATGCACATGG TGCACATGC	*agr* typing, fwd	(21)
*agr*1$_{Sa}$-2	GTCACAAGTACTATAAGCTGCGAT	*agr1* typing, rev	(21)
agr2$_{Sa}$-2	TATTACTAATTGAAAAGTGCCATAGC	*agr2* typing, rev	(21)
*agr*3$_{Sa}$-2	GTAATGTAATAGCTTGTATAATAATACCCAG	*agr3* typing, rev	(21)
*agr*4$_{Sa}$-2	CGATAATGCCGTAATACCCG	*agr4* typing, rev	(21)
arcC-Up	TTGATTCACCAGCGCGTATTGTC	*arcC*, fwd MLST typing	(9)
arcC-Dn	AGGTATCTGCTTCAATCAGCG	*arcC*, rev MLST typing	(9)
aroE-Up	ATCGGAAATCCTATTTCACATTC	*aroE*, fwd MLST typing	(9)
aroE-Dn	GGTGTTGTATTAATAACGATATC	*aroE*, rev MLST typing	(9)
β2	ATTGCCTTGATAATAGCCTTCT	*ccr1-3* typing, fwd	(12)
β4.2	TTGCGACTCTCTTGGCGTTT	*ccr4* typing, rev	(19)
blaZ-F	CAGTTCACATGCCAAAGAG	*blaZ*, PCR B, fwd	(29)
blaZ-R	TACACTCTTGGCGGTTTC	*blaZ*, PCR B, rev	(29)
blaZ-F1	GATAAGAGATTTGCCTATGC	*blaZ*, PCR C, fwd	(26)
blaZ-R1	GCATATGTTATTGCTTGACC	*blaZ*, PCR B, rev	(26)
γF	CGTCTATTACAAGATGTTAAGGATAAT	*ccr5* typing, fwd	(13)
γR	CCTTTATAGACTGGATTATTCAAAATAT	*ccr5* typing, rev	(13)
glpF-Up	CTAGGAACTGCAATCTTAATCC	*glpF*, fwd MLST typing	(9)
glpF-Dn	TGGTAAAATCGCATGTCCAATTC	*glpF*, rev MLST typing	(9)
gmk-Up	ATCGTTTTATCGGGACCATC	*gmk*, fwd MLST typing	(9)
gmk-Dn	TCATTAACTACAACGTAATCGTA	*gmk*, fwd MLST typing	(9)
gyr297	TTAGTGTGGGAAATTGTCGATAAT	*gyrB*, fwd	(34)
gyr574	AGTCTTGTGACAATGCGTTTACA	*gyrB*, rev	(34)
pta-Up	GTTAAAATCGTATTACCTGAAGG	*pta*, fwd MLST typing	(9)
pta-Dn	GACCCTTTTGTTGAAAAGCTTAA	*pta*, rev MLST typing	(9)
tpi-Up	TCGTTCATTCTGAACGTCGTGAA	*tpi*, fwd MLST typing	(9)
tpi-Dn	TTTGCACCTTCTAACAATTGTAC	*tpi*, rev MLST typing	(9)
yqiL-Up	CAGCATACAGGACACCTATTGGC	*yqiL*, fwd MLST typing	(9)
yqiL-Dn	CGTTGAGGAATCGATACTGGAAC	*yqiL*, rev MLST typing	(9)

Fwd, forward; rev, reverse

Figure 1

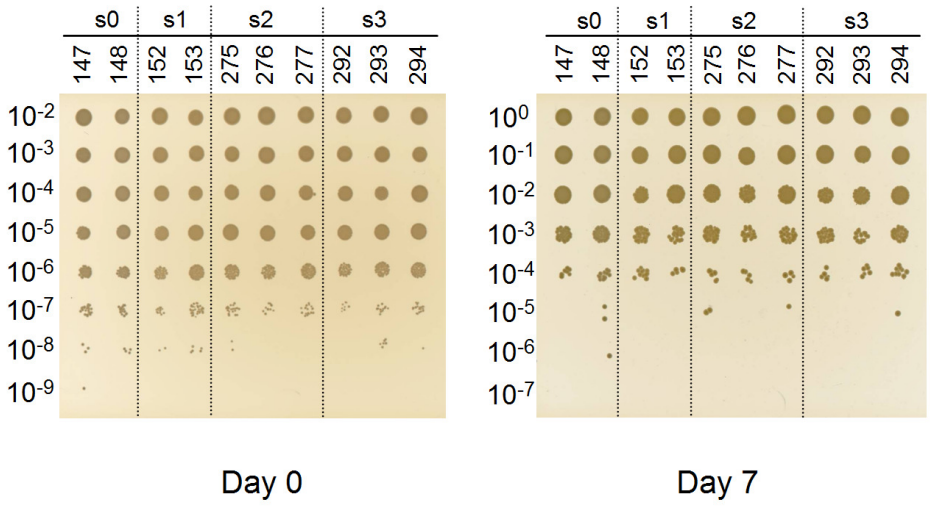

Figure 1. Desiccation resistance of volunteer 21 isolates. Isolates (ARD numbers) sampled before the administration of amoxicillin (s0) and after one week (s1), one month (s2), and two months (s3) of administration, were analyzed regarding their desiccation tolerance. Overnight cultures were dispensed into microtiter dishes and dried. After 7 days, the dried cultures were resuspended in LB, serially diluted as indicated in the figure, spotted on agar plates and compared to the initial inoculum. Representative data from two independent experiments are shown.

Figure 2

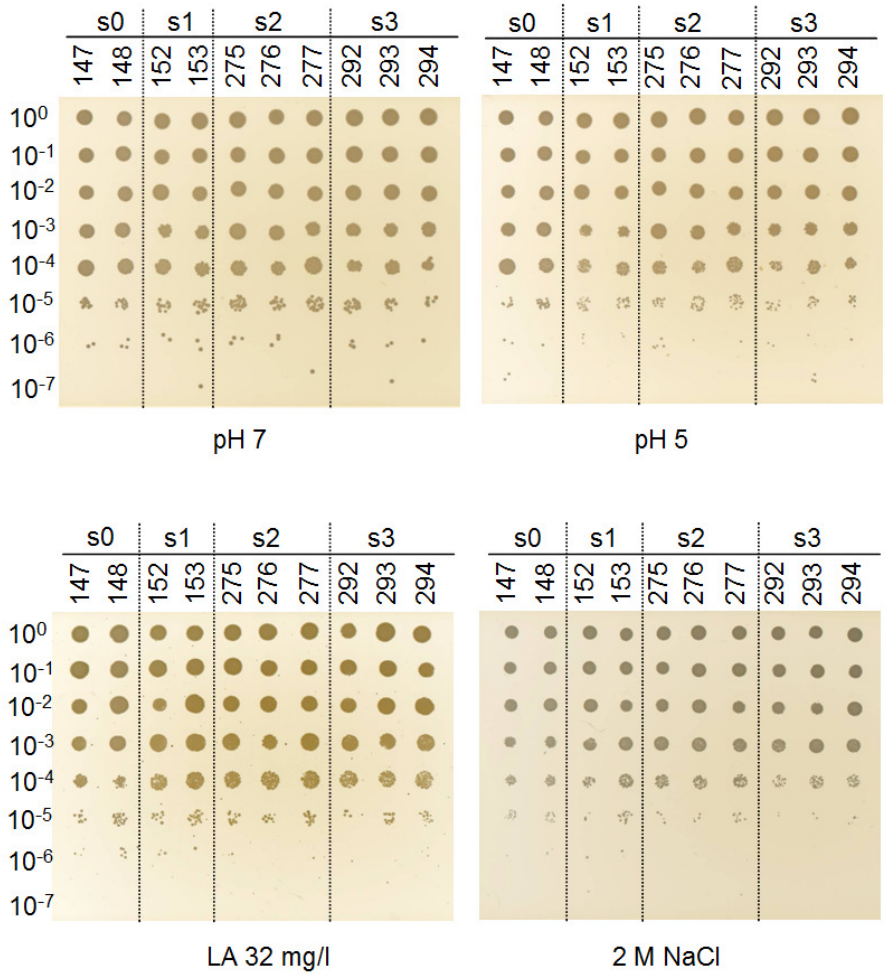

Figure 2. Stress tolerance of volunteer 21 isolates. Isolates (ARD numbers) sampled before the administration of amoxicillin (s0) and after one week (s1), one month (s2), and two months (s3) of administration, were analyzed regarding their tolerance to high salt concentrations, low pH and the presence of linoleic acid. Overnight cultures were dispensed into microtiter dishes, diluted up to 10^{-7} and spotted on LB agar adjusted to pH 7, pH 5, containing 32 mg/l linoleic acid (LA) or 2 M NaCl. Representative data from two independent experiments are shown.

Figure 3

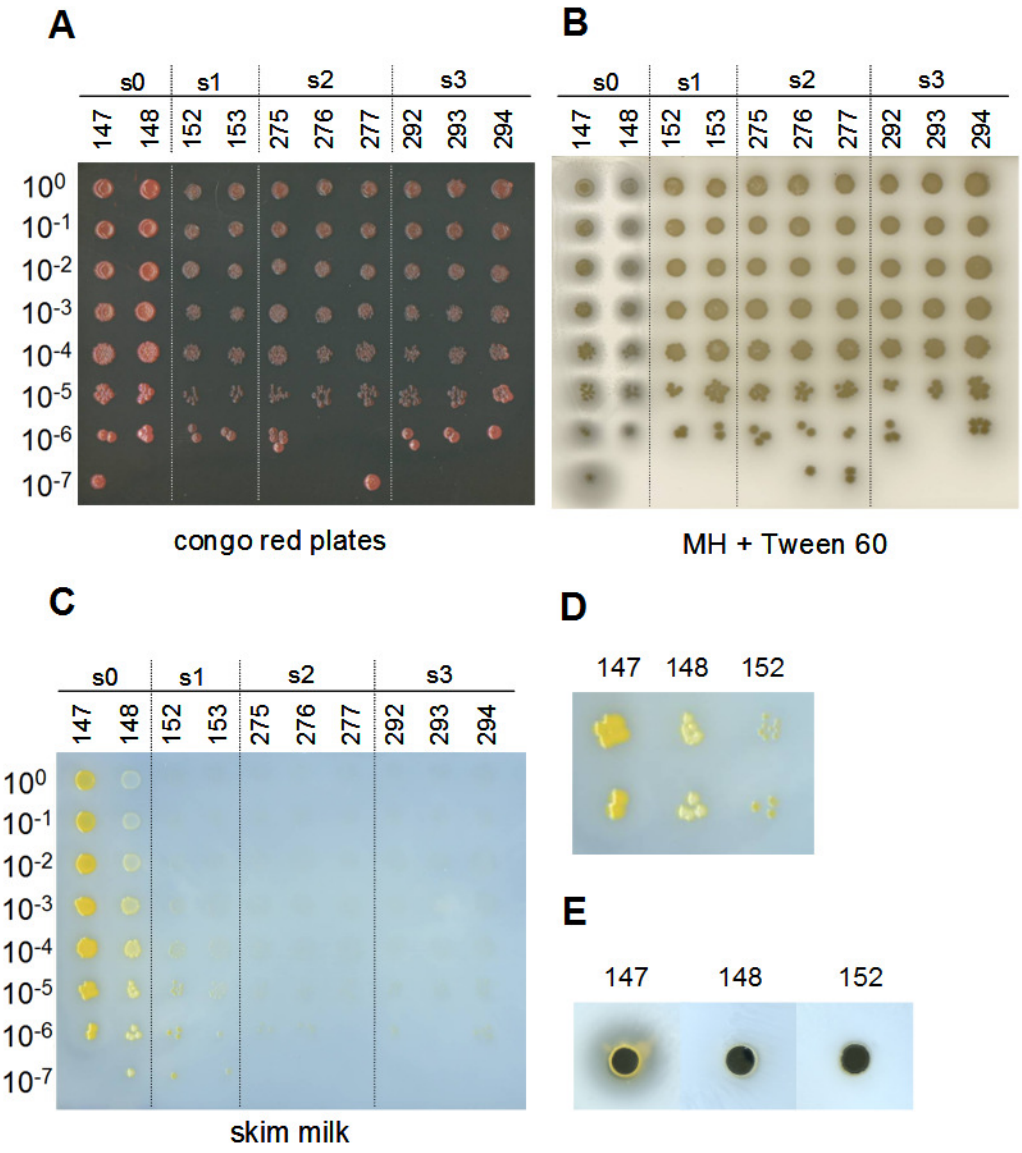

Figure 3. Extracellular factors produced by volunteer 21 isolates. Isolates (ARD numbers) sampled before the administration of amoxicillin (s0) and after one week (s1), one month (s2), and two months (s3) of administration, were analyzed regarding their production of extracellular carbohydrates, lipases and proteases. Overnight cultures were dispensed into microtiter dishes, diluted up to 10^{-7} and spotted on BHI-Congo red agar (A), MH-Tween 60 (B) or skim milk agar plates (C). Blow up of dilutions of strains ARD147, ARD148 and ARD152 on skim milk plates (D). For better visualization of proteases, OD_{600} 1 suspensions were dispensed into holes in skim milk plates (E). Representative data from at least two independent experiments are shown.

Figure 4

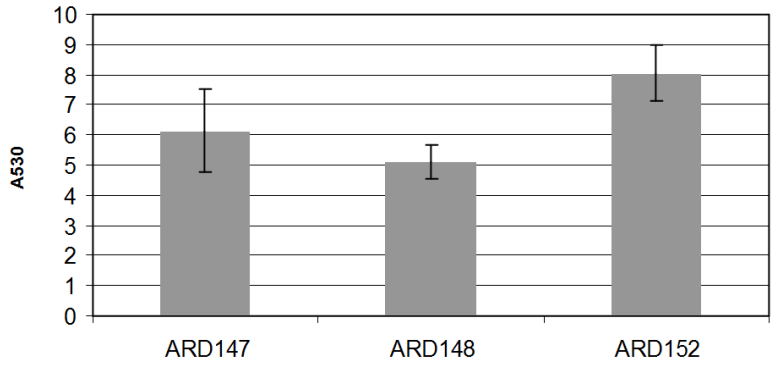

Figure 4. Biofilm produced by volunteer V021 isolates. Isolates (ARD numbers) sampled before the administration of AMX (ARD147 and ARD148) and after one week (ARD152) were tested for biofilm formation on polystyrene plates. Adhering bacteria were stained with a 0.1 % safranin solution and the color intensities were quantified by measure absorption at 530 nm (A530). Means and standard deviations from three independent experiments are shown.

Figure 5

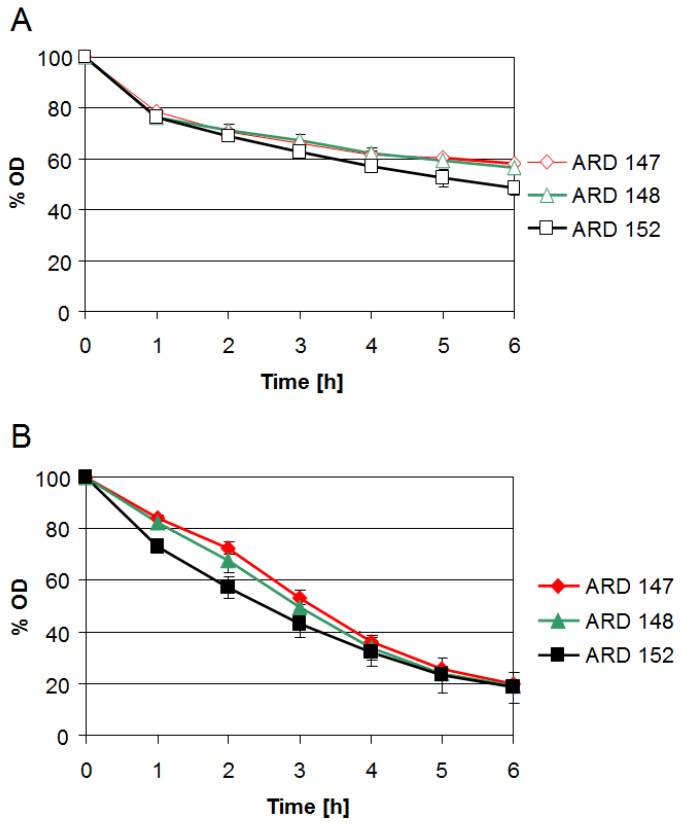

Figure 5. Spontaneous and induced autolysis of volunteer V021 isolates. Strains ARD 147 (red diamonds), ARD148 (green triangles) and ARD152 (black squares) were grown to an OD600 0.5, washed and resuspended in low salt buffer. Suspensions were split and spontaneous autolysis (A, empty symbols) or induced autolysis with 0.01 % Triton X-100 (B, filled symbols) is shown. The autolysis rate was monitored by measuring the OD over time and is given in % of the initial OD. Means and standard deviations from three independent experiments are shown.

Figure 6

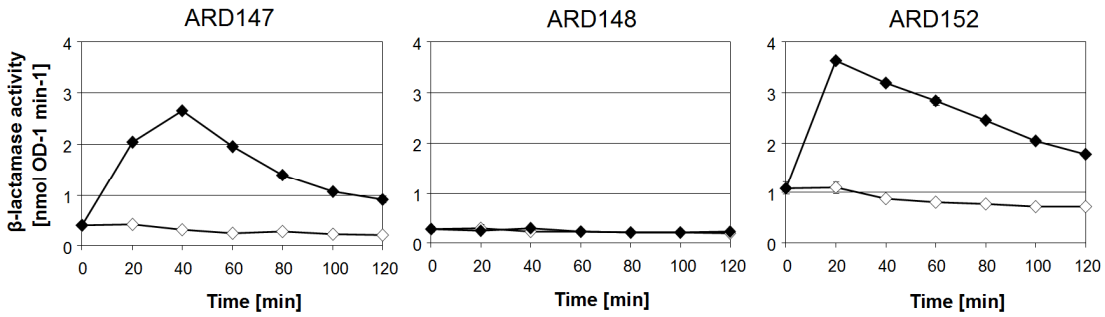

Figure 6. Beta-lactam hydrolysis by isolates ARD147, ARD148 and ARD152. Strains were grown to an OD 0.5 and induced with 0.5 MIC AMX (filled symbols) or grown without AMX (empty symbols). Samples were taken every 20 min during two hours and beta-lactamase activity was determined by measuring hydrolysis of the chromogenic cephalosporin chromacef (Sopharmia Inc., St. Joseph, USA). Representative data from three independent experiments are shown where mean values and standard deviations from three technical replicates were determined.

Figure 7

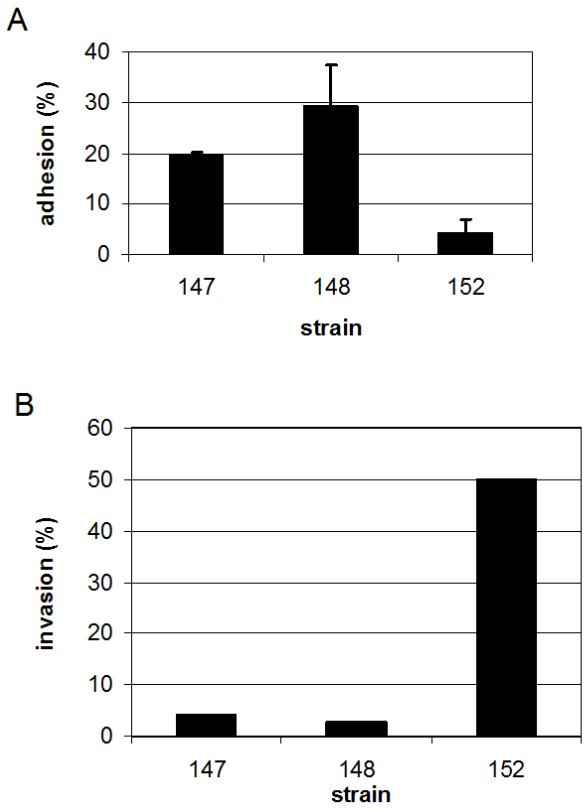

Figure 7. Adhesion, internalization and survival of V021 isolates in A549 cell cultures. Overnight cultures of strains ARD147, ARD148 and ARD152 were diluted in cell culture medium and added to confluent layers of A549 cells. Bacteria were allowed to adhere to lung epithelial cells for one hour, after which the amount of adhering bacteria was expressed in % of the initial inoculum (A). Mean values and standard deviations from three independent experiments. Invasion of lung epithelial cells was determined by measuring intracellular bacteria after 3 hours of incubation with lung epithelial cells and is given as % of the inoculum after 3 hours incubation in cell culture media (B). Representative data from three independent experiments are shown where.

4 Outlook

The factors analysed in this project are interesting candidates for the possible development of drug adjuvants that could decrease cell wall antibiotic resistance and thereby increase efficacy of existing antibiotics. This study provides new insights into the important VraSR stress response to cell wall active antibiotics, the role of LCP proteins and the relevance of c-di-AMP for fitness and resistance of MRSA. Standard administration of amoxicillin or minocycline was found to have no significant effect on fitness and resistance of commensal *S. aureus* strains in healthy volunteers. There are still many unanswered questions for possible follow-up experiments on all projects:

Even though there have been several studies on the VraSR signal transduction, the mechanism or the molecule inducing the sensor VraS is still unknown. A general stress signal for cell envelope disturbance was suggested that would be an interesting starting point for further investigation. Interesting is also follow-up investigation of the third component VraT and its role in signal transduction.

For the investigation of LCP proteins the next step would be an enzymatic confirmation of the WTA ligase function of these proteins in *S. aureus*. Whole genome sequencing of the LCP triple mutant and the corresponding wild type strain MSSA1112 could reveal possible compensatory mutations allowing its survival, which could give insights into the essentiality of the LCP protein family in *S. aureus*.

The knowledge on c-di-AMP is currently very limited and research on c-di-AMP is especially interesting since c-di-AMP is essential for bacterial growth, lowered c-di-AMP levels reduce beta-lactam resistance and c-di-AMP is universally present in bacteria but absent in humans, making it a promising drug target. The current knowledge on c-di-AMP targets and receptor proteins cannot explain all phenotypes connected to altered c-di-AMP levels, suggesting more targets could exist. C-di-AMP targets could be identified by general approaches, like proteomics, transcriptomics, metabolomics as well as pull down experiments with biotinylated c-di-AMP. c-di-AMP influences virulence and secreted c-di-AMP from *L. monocytogenes* stimulates a type I interferon response. A follow-up project could analyse the contribution of c-di-AMP to virulence in *S. aureus* and investigate if c-di-AMP is secreted by *S. aureus*.

The effect of amoxicillin and minocycline administration on the *S. aureus* population of healthy volunteers could be further validated using a larger number of *S. aureus* carriers and increasing frequency of sampling points to enhance the significance. Interesting would also be to compare the effect on volunteers from different countries that vary in their commensal *S. aureus* population.

5 References

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6 Appendix

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6. 2 Curriculum vitae

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09/2008 – 09/2009	Master studies in Biology, Major in Microbiology and Immunology, ETH Zurich Master thesis at the Institute of Molecular Biology, University of Zurich, group of Prof. Dr. Bernhard Dichtl "Analysis of the transcription termination factor Nrd1 in *Saccharomyces cerevisiae*"
02/2008 – 07/2008	Exchange semester at the University of New South Wales, Sydney, Australia
10/2007 – 02/2008	Research training at the Department of Wood Protection and Biotechnology, Laboratory of Microbiology, Empa St. Gallen
10/2004 – 09/2007	Bachelor studies in Food Sciences, ETH Zurich Bachelor thesis at the Institute of Food, Nutrition and Health, group of Prof. Dr. Martin Loessner ETH Zurich "Overexpression of *comK* in *Listeria monocytogenes* using the *flaA* promoter"
08/2000 – 09/2004	Kantonschule St. Gallen (high school), Major in Biology and Chemistry

PUBLICATIONS

Dengler, V., N. McCallum, P. Kiefer, P. Christen, A. Patrignani, J. A. Vorholt, B. Berger-Bächi and M. M. Senn. 2013. Mutation in the c-di-AMP Cyclase *dacA* Affects Fitness and Resistance of Methicillin Resistant *Staphylococcus aureus*. PLoS ONE 8: e73512.

Dengler, V., P. Stutzmann Meier, R. Heusser, P. Kupferschmied, J. Fazekas, S. Friebe, S. Burger Staufer, P. A. Majcherczyk, P. Moreillon, B. Berger-Bächi and N. McCallum. 2012. Deletion of hypothetical wall teichoic acid ligases in *Staphylococcus aureus* activates the cell wall stress response. FEMS Microbiol Lett 333:109-120.

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Chan, Y, M. Frankel, **V. Dengler**, O. Schneewind and D. Missiakas. 2013. *Staphylococcus aureus* mutants lacking the LytR-CpsA-Psr (LCP) family of enzymes release wall teichoic acids into the extracellular medium. J. Bacteriol. Aug 9. In press.

Knief, C., **V. Dengler**, P. L. Bodelier and J. A. Vorholt. 2012. Characterization of *Methylobacterium* strains isolated from the phyllosphere and description of *Methylobacterium longum* sp. nov. Antonie Van Leeuwenhoek 101:169-183.

Schubert, M., **V. Dengler**, S. Mourad and F. W. Schwarze. 2009. Determination of optimal growth parameters for the bioincising fungus *Physisporinus vitreus* by means of response surface methodology. J Appl Microbiol 106:1734-1742.

ATTENDED CONFERENCES AND WORKSHOPS

Annual Meeting of the Swiss Society for Microbiology, June 26-27, 2013, Interlaken, Switzerland. Slide presentation: “Mutation in the c-di-AMP Cyclase *dacA* Affects Fitness and Resistance of MRSA”. **V. Dengler**, N. McCallum, P. Kiefer, P. Christen, A. Patrignani, J. A. Vorholt, B. Berger-Bächi and M. M. Senn.

Interscience Conference on Antimicrobial Agents and Chemotherapy, September 9-12, 2012, San Francisco, USA. Slide presentation: “Identification of novel factors affecting methicillin resistance and the cell wall stress stimulon in *Staphylococcus aureus*”. **V. Dengler**, M. M. Senn, C. Quiblier, B. Berger-Bächi and N. McCallum.

Joint Annual Meeting of the Swiss Society for Infectious Diseases, the Swiss Society for Hospital Hygiene, the Swiss Society for Microbiology and the Swiss Society of Tropical Medicine and Parasitology, June 21-22, 2012, St. Gallen, Switzerland. Poster: “Deletion of LytR-CpsA-Psr proteins in *Staphylococcus aureus* activates the cell wall stress response and virtually abolishes the wall teichoic acid content”. **V. Dengler**, P. Stutzmann Meier, R. Heusser, S. Burger Staufer, B. Berger-Bächi and N. McCallum.

Life Science Zurich Graduate School Student Retreat, September 4-6, 2011, Chandolin, Switzerland. Slide presentation: “Deletion of LytR-CpsA-Psr proteins in *Staphylococcus aureus* activates the cell wall stress response”. **V. Dengler**, B. Berger-Bächi and N. McCallum.

Gorden Reseach Conference for Staphylococcal Dieseases, June 24-29, 2011, Lucca, Italy. Poster: “Deletion of LytR-CpsA-Psr proteins in *Staphylococcus aureus* activates the cell wall stress response“. **V. Dengler**, P. Stutzmann Meier, R. Heusser, S. Burger Staufer, B. Berger-Bächi and N. McCallum.

10th Clinical Research Day 2011, June 9, 2011, University Hospital Zurich, Switzerland. Poster: “Monitoring *in vivo* development of antimicrobial resistance in *Staphylococcus aureus*“. **V. Dengler**, M. M. Senn, B. Berger-Bächi and N. McCallum.

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International Symposium on Staphylococci and Staphylococcal Infections, September 6-9, 2010, Bath, UK. Poster: “Induction kinetics of the *Staphylococcus aureus* cell wall stress stimulon in response to different cell wall active antibiotics“. **V. Dengler**, P. Stutzmann Meier, R. Heusser, B. Berger-Bächi and N. McCallum.

Annual Meeting of the Swiss Society for Microbiology, June 24-25, 2010, Zurich, Switzerland. Posters: “Induction kinetics of the cell wall stress stimulon of *Staphylococcus aureus* using different antibiotics“. **V. Dengler**, R. Heusser, B. Over, R. Ilinca, B. Berger-Bächi and N. McCallum

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6. 3 Additional publication

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Staphylococcus aureus Mutants Lacking the LytR-CpsA-Psr (LCP) Family of Enzymes Release Wall Teichoic Acids into the Extracellular Medium

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ABSTRACT

The LytR-CpsA-Psr (LCP) proteins are thought to transfer bactoprenol-linked biosynthetic intermediates of wall teichoic acid (WTA) to the peptidoglycan of Gram-positive bacteria. In *Bacillus subtilis*, mutants lacking all three LCP enzymes do not deposit WTA in the envelope, while *Staphylococcus aureus* Δ*lcp* mutants display impaired growth and reduced levels of envelope phosphate. We show here that the *S. aureus* Δ*lcp* mutant synthesized WTA yet released ribitol-phosphate polymers into the extracellular medium. Further, Δ*lcp* mutant staphylococci no longer restricted the deposition of LysM-type murein hydrolases to cell division sites, which was associated with defects in cell shape and increased autolysis. Mutations in *S. aureus* WTA synthesis genes (*tagB*, *tarF* or *tarJ2*) inhibit growth, which is attributed to the depletion of bactoprenol, an essential component of peptidoglycan synthesis (lipid II). The growth defect of *S. aureus tagB* and *tarFJ* mutants was alleviated by inhibition of WTA synthesis with tunicamycin, whereas the growth defect of the Δ*lcp* mutant was not relieved by tunicamycin treatment nor by mutation of *tagO*, whose product catalyzes the first committed step of WTA synthesis. Further, sortase A-mediated anchoring of proteins to peptidoglycan, which also involves bactoprenol and lipid II, was not impaired in the Δ*lcp* mutant. We propose a model whereby the *S. aureus* Δ*lcp* mutant, defective in tethering WTA to the cell wall, cleaves WTA synthesis intermediates, releasing ribitol-phosphate into the medium and recycling bactoprenol for peptidoglycan synthesis.

INTRODUCTION

The peptidoglycan layer protects Gram-positive bacteria from osmotic lysis and serves as a barrier against membrane toxic compounds (1). Peptidoglycan also functions as a scaffold for the immobilization of capsular polysaccharides (2), wall teichoic acids (WTA) (3) and proteins (4). Surface proteins are anchored by sortase A, a membrane embedded transpeptidase that scans secreted polypeptides for the LPXTG motif of sorting signals (5). Sortase A cleaves the peptide bond between the threonine and the glycine of the LPXTG motif to form a thioester-linked intermediate between the carboxyl group of threonine at the C-terminal end of surface proteins and its active site cysteine residue (5, 6). The sortase A acyl intermediate is resolved by the nucleophilic attack of the free amino group of the pentaglycine crossbridge within lipid II (7, 8), the substrate for peptidoglycan biosynthesis (9). Surface protein linked to lipid II is incorporated into the cell wall envelope via the transpeptidation and transglycosylation reactions of peptidoglycan synthesis (10-12). In *Staphylococcus aureus*, lipid II is comprised of C_{55}-PP-MurNAc-(D-Ala-D-iGln-(NH_2-Gly_5)-L-Lys-D-Ala-D-Ala)-GlcNAc, i.e. a murein disaccharide-pentapeptide subunit linked to the membrane carrier bactoprenol (C_{55}). In Gram-positive bacteria, bactoprenol/undecaprenyl is also used for the synthesis of extracellular polymers such as wall teichoic acid (WTA) (13), teichuronic acid (14) and cell wall polysaccharides (15).

S. aureus WTA is a polymer of 30 to 50 ribitol-phosphate (Rbo-P) subunits connected via 1,5-phosphodiester bonds (16). Rbo-P_n is tethered to peptidoglycan via the murein linkage unit, GlcNAc-ManNAc-(Gro-P)$_{2-3}$ (17). Synthesis of the murein linkage unit is initiated by TagO (also referred to as TarO), which links UDP-GlcNAc and undecaprenyl-phosphate to generate C_{55}-PP-GlcNAc (14, 18, 19). The other WTA subunits are added to the undecaprenyl-linked intermediate via the enzymes TagA (ManNAc)(17, 20), TagBDF (Gro-P) and TarILJ (Rbo-P) (21-23). The product of this pathway, C_{55}-PP-GlcNAc-ManNAc-(Gro-P)$_{2-3}$-(Rbo-P)$_{30-50}$, is presumably flipped across the plasma membrane by the TagGH transporter (24). Attachment of the WTA polymer to the C6-hydroxyl of *N*-acetylmuramic acid within peptidoglycan [MurNAc-P-GlcNAc-ManNAc-(Gro-P)$_{2-3}$-(Rbo-P)$_{30-50}$] occurs during cell wall assembly (25). The first two genes of the WTA pathway (*tagO tagA*) can be deleted without abolishing staphylococcal growth (20, 26, 27). In contrast, *tagBDFGHtarIJL* cannot be deleted unless staphylococci carry inactivating mutations in *tagA* or *tagO* (28). This synthetic viable phenotype is explained as the limited availability of bactoprenol and its undecaprenyl-phosphate derivatives (C_{55}-PP and C_{55}-P) for peptidoglycan cell wall biosynthesis (28). WTA synthesis is blocked by tunicamycin, an antibiotic from *Streptomyces clavuligerus* that inhibits TagO (29).

Kawai *et al.* proposed that a family of genes encoding the LytR-CpsA-Psr (LCP) proteins catalyzes attachment of the murein linkage unit of WTA to the peptidoglycan of *Bacillus subtilis* (30). *B. subtilis*

encodes three *lcp* gene homologues, designated *tagTUV*, which are positioned within gene clusters for WTA biosynthetic enzymes (30). Simultaneous deletion of all three genes (*tagTUV*) is not compatible with bacterial growth unless bacilli lack the *tagO* gene. Blocking the expression of *tagTUV* causes a concomitant decrease in the synthesis of WTA (30). Further, X-ray crystallography identified polyprenyl-phosphate bound to recombinant *B. subtilis* TagT or its *S. pneumoniae* Cps2A homologue, both of which also exert *in vitro* phosphatase activity (30, 31). On the basis of these observations, Kawai *et al.* proposed that LCP enzymes recognize WTA or capsular polysaccharide synthesis intermediates as substrates for the formation of phosphodiester linkages formed between the C6-hydroxyl of MurNAc in peptidoglycan and GlcNAc-ManNAc murein linkage units (30). In agreement with this model, mutants (Δ*lcp*) lacking all of the three LCP homologues of *S. aureus* – *lcpA* (*mrsR*), *lcpB* (SA0908), and *lcpC* (SA2103) – do not harbor phosphate residues in the cell wall envelope (32) and cannot properly place cell division septa (33). Previous work left unresolved whether *S. aureus* Δ*lcp* mutants are defective in the synthesis and/or the cell wall anchoring of WTA and whether their associated cell division defect is due to the sequestration of lipid II within the WTA pathway (32, 33). The present study was performed to address these questions.

MATERIALS AND METHODS

Bacterial strains, bacterial growth and reagents.

S. aureus strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) supplemented with appropriate antibiotics. Erythromycin (Erm) and chloramphenicol (Cm) were used at a concentration of 10 µg/ml, and tunicamycin (Tun), unless otherwise specified, at 1 µg/ml. To examine the effect of tunicamycin on bacterial growth, overnight cultures grown in the absence of tunicamycin were diluted (1:100) into 100 µl fresh TSB with or without tunicamycin and growth at 37°C was monitored every 15 min for 12 hours in a Synergy HT plate reader (BioTek) by measuring the absorbance at 600 nm (A_{600}). To assess cell viability, overnight cultures of candidate strains were started by inoculating an isolated colony from a fresh plate into fresh medium. The next day, a sub-culture was prepared by diluting an aliquot of the overnight culture in tubes containing fresh medium (1:100). The tubes were placed at 37°C with shaking. Sample aliquots were removed from cultures after 3.5 h or 16 h, A_{600} values were recorded and aliquots were serially diluted and plated on agar. Colony forming units (CFU) were enumerated following incubation of plates at 37°C overnight. Bacterial strains and plasmids utilized in this study are listed in Table 1. Mutations in the three LCP-encoding genes *msrR, sa0908* and *sa2103* have been described earlier (32). For convenience, the genes are designated herein as *lcpA, lcpB* and *lcpC*, respectively. The nomenclature of single, double and triple mutants has been changed accordingly: Δ*lcpA* (*msrR*), Δ*lcpB* (*sa0908*), Δ*lcpC* (*sa2103*), Δ*lcpAB* (*msrR*/*sa0908*), Δ*lcpAC* (*msrR*/*sa2103*), and Δ*lcpBC* (*sa0908*/*sa2103*) refer to each of the single and double mutants. The designation Δ*lcp* is used to refer to the triple mutant lacking all three

lcp genes (*msrR*/*sa0908*/*sa2103*). Deletion of *tagO* (Δ*tagO*) was achieved by allelic replacement using plasmid pKOR1 (34, 35). Transposon mutagenesis was performed using two plasmids, pFA545 and pBursa (36). Transposon mutants were isolated on agar plates containing tunicamycin and candidate clones were re-screened for tunicamycin-dependent growth. To analyze the anchor structure of surface proteins, *S. aureus* strains were transformed with plasmid pHTT4 (10), which provides for the expression of the hybrid protein Seb-MH_6-Cws, where Seb and Cws represent secreted staphylococcal enterotoxin B and the cell wall sorting signal of protein A, respectively, separated by the engineered methionine-six histidine (MH_6) affinity tag for purification.

Purification of Seb-MH_6-Cws and analysis of C-terminal anchor peptides.

S. aureus MSSA1112 strains (wild-type and Δ*lcp* mutant) harboring pHTT4 (10), were grown to A_{600} 0.8. Cells were washed and suspended in 50 ml of 50 mM Tris-HCl buffer (pH 7.5), supplemented with 5 mM phenylmethanesulfonyl fluoride (PMSF) and broken in a Bead-Beater instrument (Biospec Products Inc.). Crude lysates were centrifuged at 33,000 ×*g* for 15 min and sedimented material was suspended in 100 ml of 100 mM KH_2PO_4 (pH 7.5), containing 1% Triton X-100 and 1 mM PMSF. Samples were incubated for 3 h at 4°C with stirring to extract membrane lipids. Cell wall material containing Seb-MH_6-Cws was sedimented by centrifugation at 33,000 ×*g* for 15 min, washed three times with 100 ml of 100 mM sodium phosphate buffer (pH 6.0) and suspended in 30 ml of 100 mM Tris-HCl buffer (pH 7.5) supplemented with 1 mM PMSF. Seb-MH_6-Cws was purified as described previously (10, 37). Briefly, the sample was incubated with 2 mg of lysostaphin for 16 h at 37°C and centrifuged at 33,000 ×*g* for 15 min. The supernatant containing Seb-MH_6-Cws was applied to gravity flow columns packed with Ni-NTA beads. The column was washed with 30 column volumes of buffer A (100 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl) containing 10 mM imidazole. Seb-MH_6-Cws was eluted in buffer A with 0.5 M imidazole. Protein in the eluate was precipitated with methanol/chloroform, dried under vacuum and solubilized in 3 ml 70% formic acid prior to the addition of a cyanogen bromide crystal. Samples were incubated for 16 h at room temperature in the dark. Reaction products were washed twice with water, suspended in 1 ml of buffer B (10 mM Tris-HCl, 100 mM NaH_2PO_4, 6 M guanidine hydrochloride, pH 8.0) and applied to a gravity flow column packed with Ni-NTA beads equilibrated in buffer B. The column was washed with 10-volumes each of buffer B, buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl) at pH 8.0, and buffer C at pH 6.3. Anchor peptides including H_6-Cws were eluted in buffer C containing 0.5 M imidazole at pH 8.0. For mass spectrometry analysis, peptides were desalted onto C-18 matrix cartridges (Waters) that were pre-washed with 10 ml of acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (TFA), and 10 ml of 0.1% TFA in water. Peptides were eluted in 3 ml 60% CH_3CN, 0.1% TFA, dried under vacuum and suspended in 50 µl 30% CH_3CN, 0.1% TFA. A 0.5 µl aliquot of this suspension was co-spotted with 0.5 µl of matrix, α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% CH_3CN, 0.1% TFA). Samples

were subjected to MALDI-TOF mass spectrometry using the Autoflex Speed Bruker MALDI instrument. Ions were detected in linear positive mode.

Wall teichoic acid preparations.

WTA was extracted from murein sacculi as described (38). Briefly, cells from a 30-ml culture of *S. aureus* grown in TSB at 37°C to A_{600} 1.0 were washed with 30 ml of buffer D [50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5], and suspended in 30 ml of buffer D containing 4% sodium dodecyl sulfate (SDS), followed by boiling for 1 h in a water bath and centrifugation for 10 min at 23,000 ×*g*. The sediment was washed twice in 1 ml buffer D containing 4% SDS, once in buffer D containing 2% NaCl, and once in buffer D. Following each wash, murein sacculi were sedimented by centrifugation 23,000 ×*g* for 10 min, and, after the final wash, the peptidoglycan was suspended in 1 ml of 20 mM Tris-HCl buffer at pH 8.0, containing 0.5% SDS, 20 µg proteinase K and incubated at 50°C for 4 h. Protease digested murein sacculi were washed once in buffer D containing 2% NaCl, thrice in water and suspended in 1 ml of 0.1 M NaOH for a 16-h incubation at room temperature with rotation, to hydrolyze WTA. Following sedimentation of NaOH-extracted murein sacculi at 23,000 ×*g* for 10 min, the supernatant containing the released WTA was transferred to a new tube and neutralized with 50 µl of 0.1 M acetic acid and 100 mM Tris-HCl pH 8.5 for subsequent analysis by polyacrylamide gel electrophoresis (PAGE). WTA was also precipitated from 100 ml culture supernatants of *S. aureus* grown in TSB at 37°C to A_{600} 0.5 by adding three volumes of 95% ethanol and incubating at 4°C for 30 min. Precipitated material was sedimented by centrifugation (16,000 ×*g* for 15 min), washed with 70% ethanol, air-dried and suspended in 5 ml of 100 mM Tris-HCl (pH 7.5), containing 5 mM $CaCl_2$, 25 mM $MgCl_2$, DNase (10 µg/ml) and RNase (50 µg/ml). Samples were incubated for 3 h at 37°C and subjected to methanol/chloroform extraction. TA in the aqueous layer was dried under vacuum, and solubilized in 1 ml of 20 mM Tris-HCl (pH 8.0).

Quantifying the phosphate content of the staphylococcal envelope.

Murein sacculi were prepared as described previously and stored in water (39). The phosphate content was determined by incubating 45 µl of murein sacculi sample (A_{600} 1.0) with 5 µl of trichloroacetic acid at 80°C for 16 h. Inorganic phosphate released by this treatment was quantified with a colorimetric assay where a mix composed of 6 N H_2SO_4, water, 2.5% ammonium molybdate, 10% ascorbic acid (in the ratios 1:2:1:1) was added at 1:1 (v/v) to TCA-treated preparations and incubated at 37°C for 90 min. Product formation corresponding to free phosphate was measured in a spectrophotometer at 820 nm (A_{820}) and phosphate concentration in the samples was calculated from NaH_2PO_4 standards (concentration 0-800 µM).

Gel electrophoresis and immunoblotting.

WTA was separated on polyacrylamide gels using a Bio-Rad Protean II xi electrophoresis cell (20 cm × 16 cm × 1 mm) (40). Following separation of extracts by electrophoresis, gels were incubated twice in 1mg/ml alcian blue for 20 min, followed by two 30 min washes with deionized H_2O and staining with the Silver Stain Plus Kit (Bio-Rad). For comparative analyses of proteins, aliquots of cultures used for WTA analysis were removed, treated with lysostaphin (4 µg) and incubated at 37°C for 10 min. Proteins in these lysates were precipitated with 7% trichloroacetic acid, washed once with acetone, dried, solubilized in 100 µl of 0.5 M Tris-HCl pH 8.0 containing 4% SDS and heated at 90°C for 10 min. Proteins were separated on 12% SDS-PAGE and transferred to poly(vinylidene difluoride) membrane (Millipore) for immunoblot analysis with appropriate polyclonal antibodies. Immunoreactive signals were revealed by using a secondary antibody coupled to IRDye© 680 and visualized with a Li-Cor Biosciences Odyssey imager.

Phenotypic characterization of mutants.

The ability of *S. aureus* mutants to bind purified LysM-mCherry was assessed as described previously (34, 41). Briefly, cells of logarithmically growing cultures were sedimented by centrifugation, washed in phosphate-buffered saline (PBS), and incubated with purified LysM-mCherry for 10 min. mCherry was used as a control. Following incubation, cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS prior to analysis by flow cytometry and fluorescence microscopy. Incubation with propidium iodide (Invitrogen) was used to evaluate the membrane integrity of mutants as compared to wild-type. Briefly, overnight cultures were diluted 1:100 into TSB containing 0 or 1 µg/ml tunicamycin and cultured at 37°C for 3.5 h. Following centrifugation of 1 ml culture, cells were washed twice with PBS, fixed for 20 min with 4% paraformaldehyde and then blocked for 1 h in PBS with 1% bovine serum albumin. Cells were incubated for 15 min with 10 nM SYTO 9 (Invitrogen) and 2 µg/ml propidium iodide in PBS, washed twice and suspended in PBS for flow cytometry measurements.

Flow cytometry and microscopy.

Flow cytometric analyses were performed using the BD-LSR-II cytometer. mCherry fluorescence was quantified using the allophycocyanin (APC) parameter and gating on single cells using forward and side scatter. Staphylococci were gated using forward and side scatter, and SYTO 9-positive cells captured under the FITC parameter were analyzed for propidium iodide staining in the PerCP-A parameter. The parameters for negative propidium iodide staining were determined using an unstained control. For fluorescence microscopy, images were captured on a Leica TCS SP2 AOBS laser-scanning confocal microscope with a 100× objective using identical settings and exposure times between samples. For transmission electron microscopy, bacterial cells were washed twice with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, bathed in fixative (2% glutaraldehyde, 4% PFA, 0.1 M sodium

cacodylate buffer) overnight at 4°C, and post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 60 min. Fixed samples were stained in 1% uranyl acetate in maleate buffer for 60 min, serially dehydrated with increasing concentrations of ethanol, embedded in spurr resin for 48 h at 60°C, thin sectioned (90 nm) using a Reichert-Jung Ultracut device and post-stained in uranyl acetate and lead citrate. The samples were imaged on the FEI Tecnai F30 with a Gatan charge-coupled device (CCD) digital micrograph.

RESULTS

The *S. aureus* Δ*lcp* mutant releases wall teichoic acid into the extracellular medium.

A *S. aureus* mutant with all three *lcp* genes deleted harbors very little phosphate in its cell wall envelope, suggesting that the mutant does not synthesize WTA (32). We wondered whether *S. aureus lcp* mutants may also be defective in the cell wall anchoring of WTA (30). To examine these possibilities, murein sacculi were isolated from wild-type *S. aureus* MSSA1112 and its isogenic mutants lacking one (Δ*lcpA*, Δ*lcpB* and Δ*lcpC*), two (Δ*lcpAB*, Δ*lcpAC* and Δ*lcpBC*) or all three *lcp* genes (Δ*lcp*). WTA was released from murein sacculi via alkaline hydrolysis; Rbo-P polymers of variable length were resolved by PAGE and visualized by staining with alcian blue/silver. As controls, the murein sacculi of wild-type *S. aureus* harbored WTA, whereas treatment of staphylococci with tunicamycin, an inhibitor of TagO, blocked the synthesis and incorporation of WTA in to the cell wall (Fig. 1A, lanes 1 and 2). The minimal inhibitory concentration of tunicamycin to block WTA synthesis in *S. aureus* MSSA1112 was 1 μg/ml (Fig. S1). WTA was detected in the murein sacculi of each single and double *lcp* mutant (Fig. 1A, lanes 3 to 8) but not in sacculi prepared from the Δ*lcp* strain (Fig. 1A, lane 9). This WTA synthesis defect of the Δ*lcp* mutant was restored by transformation with plasmids expressing any one of the three LCP homologues, *lcpA, lcpB,* or *lcpC* (Fig. 1B, lanes 11-13). We also determined the cell wall phosphate content of the wild-type versus mutant strains via colorimetric assay. Here, peptidoglycan was extracted and purified from exponentially grown bacteria and inorganic phosphate liberated by incubation in 10% trichloroacetic acid. Staphylococci incubated with tunicamycin synthesized and incorporated very little phosphate into the cell wall envelope (Fig. 1C). As already noted by Dengler *et al.* (32), Rbo-P was not detected in cell wall extracts derived from the Δ*lcp* mutant. Furthermore, the single mutants Δ*lcpA* and Δ*lcpB*, but not Δ*lcpC*, contained reduced levels of phosphate compared to wild-type as did each of the double mutants (Fig. 1C). Nevertheless, loss of two *lcp* genes did not cause clear synergistic effects in reducing the phosphate content of staphylococcal cell walls (Fig. 1C).

We next examined whether Rbo-P polymer can be detected in the culture medium of Δ*lcp* staphylococci. Following growth in the presence or absence of tunicamycin, carbohydrates and teichoid acid polymers in the supernatant of centrifuged cultures from wild-type MSSA1112 or its *Δlcp* variant were precipitated with ethanol. Teichoic acids were sedimented by centrifugation and

contaminating nucleic acids digested with DNase and RNase. Samples were extracted with methanol and chloroform to remove proteins. Teichoic acids in the aqueous layer were concentrated by lyophilization, suspended in water, subjected to PAGE and revealed by alcian blue/silver staining. As controls, murein sacculi of wild-type MSSA1112 harbored Rbo-P WTA, whose synthesis was blocked by tunicamycin treatment (Fig. 1D). Of note, Rbo-P polymer was detected in the extracellular medium of cultures from the Δ*lcp* mutant, but not those of wild-type *S. aureus* (Fig. 1D). Tunicamycin treatment of Δ*lcp* cultures abolished the release of the Rbo-P polymer into the culture medium (Fig. 1D). To ascertain that Rbo-P polymer release did not result from cell lysis or aberrant peptidoglycan turnover, we asked whether the Δ*lcp* mutant also released cytoplasmic proteins or other cell wall polymers such as sortase-anchored proteins in the medium. We show that the hybrid protein Seb-MH_6-Cws, with the cell wall sorting signal of protein A, is properly tethered to the murein sacculus of the Δ*lcp* mutant. Immunoblotting analyses revealed that the hybrid is not aberrantly released into the extracellular milieu of Δ*lcp* mutant cultures as compared to wild-type staphylococci (Fig. S2). The cytoplasmic protein L6 was not detected in extracts of spent culture media, ruling out bacterial lysis as a mechanism for surface protein release into the culture medium (Fig. S2). Thus, we conclude that the Δ*lcp* mutant fails to deposit WTA in the cell wall envelope, and instead releases Rbo-P into the extracellular medium. Taken together, these data suggest that LcpA, LcpB and LcpC display overlapping functions in depositing WTA in the cell wall envelope, presumably by forming a phosphodiester bond between the C-6 hydroxyl of MurNAc and the murein linkage unit tethered to Rbo-P_n.

Cell division and envelope defects of Δ*lcp* and Δ*lcp*Δ*tagO* mutant staphylococci.

In *B. subtilis*, deletion of all three *lcp* genes is not compatible with bacterial growth unless the mutant cells also harbor a deletion of the *tagO* gene (30). In contrast, the *S. aureus* Δ*lcp* mutant, that also lacks all three *lcp* genes, continues to grow (A_{600}), albeit at a slower rate than wild-type (Fig. S3). To assess the viability of staphylococcal cells, culture aliquots were removed, serially diluted and plated after 3.5 h to enumerate viable colony forming units (CFU). We observed a 3-log reduction in plating efficiency for the Δ*lcp* mutant as compared to wild-type MSSA1112 (Fig. 2A). Introduction of the Δ*tagO* allele into the Δ*lcp* mutant neither restored plating efficiency to wild-type levels, nor enhanced growth of the mutant at the earlier 3.5 h timepoint (Fig. 2A), when the LCP enzymes are predominantly expressed (42). Further, when growth was measured via absorbance of liquid cultures (A_{600}), the Δ*lcp*Δ*tagO* mutant replicated at a rate similar to the Δ*lcp* mutant, but not at the rate of its wild-type parent (Fig. S3). To measure the number of non-viable staphylococci, culture aliquots of the three strains were stained with propidium iodide to identify lysed bacteria with membrane damage via flow cytometry (Fig. 2B). These experiments revealed that 30.2% of Δ*lcp* cells and 18.7% of Δ*lcp*Δ*tagO* cells were positive for propidium iodide, compared to only 1.68% of wild-type cells (Fig.

2B). Wild-type and mutant staphylococci were fixed, thin sectioned and viewed by transmission electron microscopy. As expected, wild-type strain MSSA1112 formed round cells, with a thick cell wall envelope and cross wall septa positioned at mid cell, perpendicular to previous cell division planes (Fig. 2C). Uranyl acetate staining revealed the uniform deposition of WTA as electron dense deposits in the envelope of wild-type staphylococci (Fig. 2C) (43). In contrast, the Δ*lcp* and Δ*lcp*Δ*tagO* variants generated deformed cells with thin, irregular envelopes lacking the WTA staining of wild-type staphylococci (Fig. 2C). Of note, the Δ*lcp* and Δ*lcp*Δ*tagO* variants generated cross wall septa parallel to the previous division planes, indicating that the physiological mechanisms for the selection of cell division planes had been abolished in the Δ*lcp* and Δ*lcp*Δ*tagO* mutants (Fig. 2C). Interestingly, this phenotype is also observed for the isolated *tagO* and *lcpA* mutants, the latter of which still produces WTA (33, 43). These data suggest that both WTA synthesis and assembly are important for physiological cell division (43). In conclusion, the growth delay observed in the Δ*lcp* mutant is not a direct result of the continued synthesis of WTA and cannot be restored by preventing WTA synthesis.

Effect of tunicamycin on the generation of non-viable daughter cells by Δ*lcp* mutant staphylococci. Dengler *et al.* reported that tunicamycin treatment could partially alleviate the growth defect of Δ*lcp* mutant staphylococci, a phenotype that was also observed in our experiments (Fig. 3A). We wondered whether tunicamycin treatment also suppressed the phenotype of the Δ*lcp* mutant of non-viable daughter cells. Bacterial cultures were grown without or with 1 μg/ml of tunicamycin for 3.5 h. Culture aliquots were serially diluted and plated on TSA for colony formation (Fig. 3A). As expected, the plating efficiency of wild-type MSSA1112 was slightly reduced when the strain was grown in the presence of tunicamycin. In contrast, the addition of tunicamycin did not affect the plating efficiency of the Δ*lcp* strain (Fig. 3A), a phenotype that is in stark contrast to strains with mutational lesions in genes of the WTA biosynthetic pathway. For example, disruption of *S. aureus tagB*, *tarF* or *tarJ2* via *bursa aurealis* transposon insertion was conditional for the presence of tunicamycin in agar media, whereas the plating efficiency of the Δ*tagO* mutant was not (Fig. S3). The plating efficiency of Δ*lcp* strain and its sensitivity to tunicamycin were almost restored to wild-type levels upon transformation with plasmids that provide for the expression of any one of the three *lcp* genes (Fig. 3A). Of note, the reduction in plating efficiency was not always correlated with bacterial growth measured for wild-type and Δ*lcp* cultures via increases in A_{600} (Fig. 3A). As before, this difference can be explained by the generation of non-viable daughter cells, revealed by the increase of propidium iodide positive cells in the Δ*lcp* culture. While incubation of cultures with tunicamycin led to further increases in propidium iodide-positive Δ*lcp* cells, tunicamyin treatment did not affect the membrane integrity of wild-type staphylococci (Fig. 3B). Transmission electron microscopy revealed that tunicamycin treatment did not affect the overall morphology and cell division septum formation in wild-type cells; as expected, the outer electron dense layer of WTA was abolished when staphylococci

were grown in the presence of the antibiotic (Fig. 3C). Further, tunicamycin treatment of Δ*lcp* staphylococci did not change the aberrant gross morphology and septation defect of the mutant cells (Fig. 3C). These data show that tunicamycin treatment, an inhibitor of WTA synthesis, neither suppresses the cell division defect of Δ*lcp* staphylococci nor its generation of non-viable daughter cells.

The *lcp* genes are not required for surface protein anchoring to the staphylococcal cell wall. Previous work identified lipid II as the peptidoglycan substrate of the sortase A-catalyzed transpeptidation reaction of surface protein anchoring to the cell wall envelope (8). Consistent with this model, surface protein anchoring can be blocked by treating staphylococci with inhibitors of lipid II polymerization (vancomycin or moenomycin) or bactoprenol metabolism (nisin) (8). LCP proteins have been proposed to transfer WTA precursors [C_{55}-PP-GlcNAc-ManNAc-(Gro-P)$_{2-3}$-(Rbo-P)$_{30-50}$] onto the glycan strands of peptidoglycan (30). Since both reactions—surface protein- and WTA-anchoring—require bactoprenol-linked intermediates, we wondered whether sortase A-mediated protein anchoring is altered in *S. aureus* strains lacking the three LCP phosphotransferases (Δ*lcp* strain). To test this possibility, the wild-type parent strain MSSA1112 and the Δ*lcp* mutant were transformed with plasmid pHTT4, which provides for the expression of Seb-MH_6-Cws, an engineered surface protein consisting of the N-terminal signal peptide and staphylococcal enterotoxin B (Seb) fused to the C-terminal cell wall sorting signal of protein A (Fig. 4A). Peptidoglycan preparations from both MSSA1112 strains (WT/pHTT4 and Δ*lcp*/pHTT4) were treated with lysostaphin to solubilize anchored Seb-MH_6-Cws for purification via metal-chelating affinity chromatography (Fig. 4B). Purified proteins were subjected to cyanogen bromide (CNBr) cleavage at methionine residues. C-terminal anchor peptides with their N-terminal six-histidine (H_6) tag were again purified by affinity chromatography and analyzed by MALDI-MS (Fig. 4CD). Ion signals labeled with *m/z* 1665 [1] and *m/z* 1722 [3] correspond to surface protein anchor peptides with two or three glycine residues [H_2N-H_6AQALPETGG(G)], which are released from the cell wall via lysostaphin cleavage (Fig. 4CD, Table 2). Ion signals with *m/z* 1693 [2] and *m/z* 1750 [4] represent formylated peptides of compounds 1 and 3 (Table 2). Compounds with *m/z* 3826 [5], *m/z* 3854 [6], and *m/z* 3903 [7] represent formylated or carbamylated anchor peptides with two or three glycine residues, also liberated via lysostaphin cleavage from the cell wall [NH_2-VDSKDVKIEVYLTTKKGTMH_6AQALPETG(G)]; these peptides were generated via incomplete CNBr cleavage of Seb-MH_6-CWS at methionyl residues (Table 2). Thus, Seb-MH_6-CWS anchor peptides with similar structure and abundance were liberated via lysostaphin treatment from the cell wall envelope of wild-type and Δ*lcp* mutant staphylococci. These data indicate that the absence of LCP enzymes does not affect sortase A-mediated anchoring of surface proteins in *S. aureus*.

Deposition of LysM murein hydrolases in the envelope of Δ*lcp* mutant staphylococci.

The decoration of the staphylococcal peptidoglycan with WTA restricts the binding of secreted murein hydrolases to the cell wall and limits the autolytic activity of these enzymes to the cross wall compartment of dividing staphylococci (44). For example, the deposition of the LytN and Sle1 murein hydrolases is restricted to the cross wall (34, 41). Each of these enzymes harbors LysM domains at their N-termini (34). LysM domains are both necessary and sufficient for the targeting of Sle1 to the cross wall (34). Inhibition of WTA synthesis, for example treatment of staphylococci with tunicamycin or deletion of *tagO*, abolishes the specificity of LysM-domain targeting to the bacterial envelope (34). In a confocal fluorescence microscopy experiment, an abundance of $LysM_{LytN}$-mCherry and $LysM_{Sle1}$-mCherry was found deposited uniformly throughout the envelope of *tagO* mutant, but not wild-type staphylococci (34)(Fig. 5A). As a control, mCherry alone did not bind to the staphylococcal envelope. We wondered whether the Δ*lcp* mutant, which fails to deposit WTA in the staphylococcal envelope, is also defective for the targeting of LysM-type murein hydrolases. Similar strong binding activities of $LysM_{LytN}$-mCherry and $LysM_{Sle1}$-mCherry were observed for the Δ*lcp* mutant strain, but not for its MSSA1112 parent (Fig. 5A).

The deposition of mCherry, $LysM_{LytN}$-mCherry and $LysM_{Sle1}$-mCherry on the staphylococcal surface was quantified by measuring bacterial fluorescence in a flow cytometry experiment (Fig. 5B). A 10- and 100-fold increase in $LysM_{LytN}$-mCherry and $LysM_{Sle1}$-mCherry fluorescence, respectively, was measured with Δ*lcp* mutant staphylococci relative to the wild-type strain MSSA1112. These differences were comparable to those observed between wild-type and Δ*tagO* mutant staphylococci (Fig. 5B). As a control, mCherry did not bind to the envelope of wild-type, Δ*tagO*, or Δ*lcp* mutant staphylococci (Fig. 5AB). These data suggest that the WTA deposition defect of the Δ*lcp* mutant causes the unrestricted deposition of murein hydrolases in the bacterial envelope, a phenotype that likely contributes to the decreased viability of Δ*lcp* daughter cells generated during cell division.

DISCUSSION

Recent reports implicated a family of genes encoding the LCP proteins in WTA synthesis and in the attachment of secondary cell wall polymers to bacterial peptidoglycan (30, 31). LCP-encoding genes are present in virtually all Gram-positive bacteria, often numerous homologues within a single genome (45). The LCP proteins share a similar predicted secondary structure, consisting of a short N-terminal cytoplasmic tail, a single transmembrane domain, and an extracellular C-terminal region encompassing a mixed α-helical/β-sheet "LCP" domain (30, 45). While the biochemical activity of these proteins is still not known, variants carrying defective alleles of *lcp* genes display pleiotropic phenotypes. For example, in both *S. aureus* and *S. pneumoniae*, loss of one or more of these genes resulted in aberrant septum formation (33, 46, 47), increased susceptibility to β-lactam antibiotics (33,

46), autolysis (33), aberrant biofilm formation (46), induction of cell wall envelope stress responses (32) and reduced cell wall phosphate (32). *B. subtilis* contains three homologues of the LCP genes, *tagTUV*, which cluster with genes of the WTA synthesis pathway (30). Deletion of all three genes is not compatible with growth of the mutant bacilli (30). Similar to late-stage WTA mutants, this phenotype can be rescued through the additional deletion of *tagO* (28); furthermore, *tagTUV* mutants cannot assemble WTA in the bacterial cell wall (30). In *S. pneumoniae*, mutants with deletions of one or two of the LCP homologues (Cps2A, LytR and Psr) synthesize reduced amounts of capsular polysaccharide and release some of this material into the extracellular medium (31, 48, 49). The crystal structures of recombinant *B. subtilis* TagT and *S. pneumoniae* Cps2A revealed the presence of a polyprenyl phosphate lipid bound to the purified protein (30, 31). These findings have led to the hypothesis that LCP enzymes transfer secondary cell wall polymers from their undecaprenyl-phosphate precursors and link them to the bacterial peptidoglycan (30). Specifically, LCP enzymes are proposed to tether the murein linkage unit of WTA or other secondary cell wall polysaccharides to the C6-hydroxyl of MurNAc in the peptidoglycan of Gram-positive bacteria (30).

Although several layers of evidence support the hypothesis of Kawai and colleagues, there are also arguments against it. For example, *S. pneumoniae* is not known to express functional *tagO tagA* homologues or synthesize murein linkage units (C_{55}-PP-GlcNAc-ManNAc); pneumococci are thought to anchor their teichoic acid polymers and capsular polysaccharides to the C6-hydroxyl of MurNAc via phosphodiester bonds with other carbohydrate structures (50). Further, if *B. subtilis* TagTUV or *S. aureus* LcpABC were to tether the murein linkage units of WTA to the cell wall, one would expect that the corresponding mutant strains either harbor WTA precursors [C_{55}-PP-GlcNAc-ManNAc-Gro_{2-3}-(Rbo-P_n)] in the envelope or release unanchored products into the extracellular medium. Although this was tested, the *B. subtilis tagTUV* mutant did not release WTA into the medium and did also not deposit WTA in the envelope (30).

We are interested in sortase-mediated anchoring of surface proteins to the cell wall envelope (5), a biosynthetic pathway that also utilizes an undecaprenyl precursor, lipid II, to immobilize polypeptides in the bacterial envelope (7, 8, 51). We presumed that the *S. aureus Δlcp* mutant may accumulate undecaprenyl-linked intermediates of the WTA pathway, thereby causing a block in surface protein anchoring. This, however, was not observed; instead, the *S. aureus* Δ*lcp* mutant synthesized WTA and released the Rbo-P polymers into the extracellular medium. We surmise that the observed release of WTA may be catalyzed by undecaprenyl pyrophosphate phosphatases (UppPs), which are otherwise involved in the recycling of translocated lipid II (52). If so, this could explain why undecaprenyl recycling occurs in the Δ*lcp* variant but not in *tagB*, *tarF* or *tarJ2* mutants, which require mutations in *tagO*/*tagA* or inhibition of WTA synthesis with tunicamycin for growth. We entertain a model whereby the *S. aureus* Δ*lcp* mutant translocates its WTA precursors via TagGH for subsequent hydrolysis via UppPs, thereby enabling the return of bactoprenol into the cytoplasm. This model could

also explain why other WTA biosynthetic intermediates, those accumulating in *tagB*, *tarF* or *tarJ2* mutants (without substrate properties for TagGH), either deplete staphylococci of the lipid carrier or accumulate toxic intermediates. It seems plausible that UppPs do not have access to cytoplasmic intermediates of the WTA pathway and therefore cannot relieve the growth inhibitory attributes of *tagB*, *tarF* or *tarJ2* mutations. Our model offers insights for the development of new drugs that target key steps of WTA synthesis on the bacterial surface. At least for *S. aureus*, the causative agent of human infections with antibiotic resistant strains (53), small molecule inhibitors of LCPs would not be expected to cause bactericidal activity, whereas the combined inhibition of LCPs and UppPs may be associated with antibiotic activity. In contrast, the *B. subtilis tagTUV* mutant appears to sequester WTA synthesis intermediates, as these mutations inhibit the growth of the mutant unless WTA synthesis is blocked via *tagO* mutation (30). Thus, *B. subtilis* may lack the relevant UppP activity and cannot recycle WTA synthesis intermediates. A combined literature and Genbank search suggests that *S. aureus* encodes three genes with undecaprenyl pyrophosphate phosphatase activity. The canonical *bacA*/*uppP* gene has been described and is dispensable for growth (54). *S. aureus* also encodes two predicted phosphatases with a PAP2-domain reminiscent of *ybjG* and *pgpB*, two genes that have been shown to encode redundant UppP activities *E. coli* (52). A similar search performed for *B. subtilis* suggest that this organism encodes only two undecaprenyl pyrophosphate phosphatases, *bacA* (formerly *yubB*) and *bcrC* (formerly *ywoA*) (55).

In sum, data reported here support the model of Kawai and colleagues whereby LCP enzymes catalyze the transfer of WTA synthesis intermediates to the cell wall peptidoglycan (30). The observed pleiotrophic phenotypes of the Δ*lcp* mutant are likely due to its WTA synthesis and WTA cell wall deposition defects. Without WTA, staphylococci display aberrant cell size, septum formation, autolysis, susceptibility to antibiotics and defects in biofilm formation (32, 33, 45, 46). The molecular basis of the observed phenotypes are likely due to defects in the positioning and functional coordination of the peptidoglycan biosynthesis and cell wall separation machines, which involve a wide spectrum of different enzymes.

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Table 1. Strains and plasmids used in this study.

Strain designation	**Genotype**	**Description**	**Source**
RN4220	Wild-type	*S. aureus* laboratory strain, a restriction-deficient derivative of NCTC 8325-4	(56)
Δ*tagO*	RN4220 *tagO*	RN4220 lacking *tagO*	(57)
tagB::erm	RN4220 *tagB*	RN4220 with *bursa aurealis* insertion in *tagB*	This work
tarF::erm	RN4220 *tarF*	RN4220 with *bursa aurealis* insertion in *tarF*	This work
tarJ2::erm	RN4220 *tarJ2*	RN4220 with *bursa aurealis* insertion in *tarJ2*	This work
MSSA1112	Wild-type	Methicillin sensitive *S. aureus*, clinical isolate	(58)
Δ*lcpA*	MSSA1112 *lcpA::erm*	MSSA1112 with *ermB* replacing *lcpA (msrR)*	(46)
Δ*lcpB*	MSSA1112 *lcpB*	MSSA1112 lacking *lcpB (sa0908)*	(33)
Δ*lcpC*	MSSA1112 *lcpC*	MSSA1112 lacking *lcpC (sa2103)*	(33)
Δ*lcpAB*	MSSA1112 *lcpA/lcpB*	MSSA1112 lacking *lcpA* and *lcpB*	(33)
Δ*lcpAC*	MSSA1112 *lcpA/lcpC*	MSSA1112 lacking *lcpA* and *lcpC*	(33)
Δ*lcpBC*	MSSA1112 *lcpB/lcpC*	MSSA1112 lacking *lcpB* and *lcpC*	(33)
Δ*lcp*	MSSA1112 *lcp*	MSSA1112 lacking all three *lcp* genes	(33)
Δ*lcp*Δ*tagO*	MSSA1112 *lcp/tagO*	MSSA1112 lacking all three *lcp* genes and *tagO*	This work
Plasmid designation	**Insert**	**Description**	
pΔ*tagO*	DNA segments flanking *tagO*	pKOR1 carrying 1 kbp DNA segment upstream and downstream of *tagO* used for allelic replacement	This work
pHTT4	*SEB-MH$_6$-CWS*	pOS1 vector encoding staphylococcal enterotoxin B with the cell wall sorting motif of protein A	(10)
pBursa	Modified mariner transposon	Plasmid carrying the mariner based transposon with erythromycin resistance (thermosensitive replicon)	(36)
pFA545	Mariner transposase	Plasmid carrying the transposase (thermosensitive replicon)	(36)
p*lcpA*	*lcpA* (*msrR*)	Plasmid pGC2 (pT194-based) encoding *lcpA* for complementation studies	(33)
p*lcpB*	*lcpB* (*sa0908*)	Plasmid pGC2 (pT194-based) encoding *lcpB* for complementation studies	(33)
p*lcpC*	*lcpC* (*sa2103*)	Plasmid pGC2 (pT194-based) encoding *lcpC* for complementation studies	(33)

Table 2. Sortase A-catalyzed surface protein anchoring to the cell wall is not altered in the Δ*lcp* mutant.

Ion	WT/pHTT4 Obs. *m/z*	Δ*lcp*/pHTT4 Obs. *m/z*	Calc. *m/z*	Proposed Structure
1	1665.941	1665.811	1665.729	NH_2-H_6AQALPET-Gly_2-CO_2H
2	1693.893	1693.830	1693.739	HCO-NH-H_6AQALPET-Gly_2-CO_2H
3	1722.888	1722.827	1722.781	NH_2-H_6AQALPET-Gly_3-CO_2H
4	1750.956	1750.867	1750.791	HCO-NH-H_6AQALPET-Gly_3-CO_2H
5	3826.572	3826.436	3826.215	NH_2-VDSKDVKIEVYLTTKKGTMH_6AQALPET-Gly_2-CO_2H, Na^+
6	3854.116	3854.111	3854.225	HCO-NH-VDSKDVKIEVYLTTKKGTMH_6AQALPET-Gly_2-CO_2H, Na^+
7	3901.934	3902.469	3903.301	H_2NCO-NH-VDSKDVKIEVYLTTKKGTMH_6AQALPET-Gly_3-CO_2H

Cyanogen bromide cleavage products of cell wall anchored Seb-MH_6-Cws liberated by lysostaphin treatment and purified by Ni-NTA chromatography (see Fig. 4) were analyzed by MALDI-TOF MS in linear positive mode. The program CS ChemDraw was used for *m/z* calculations.

Figure 1

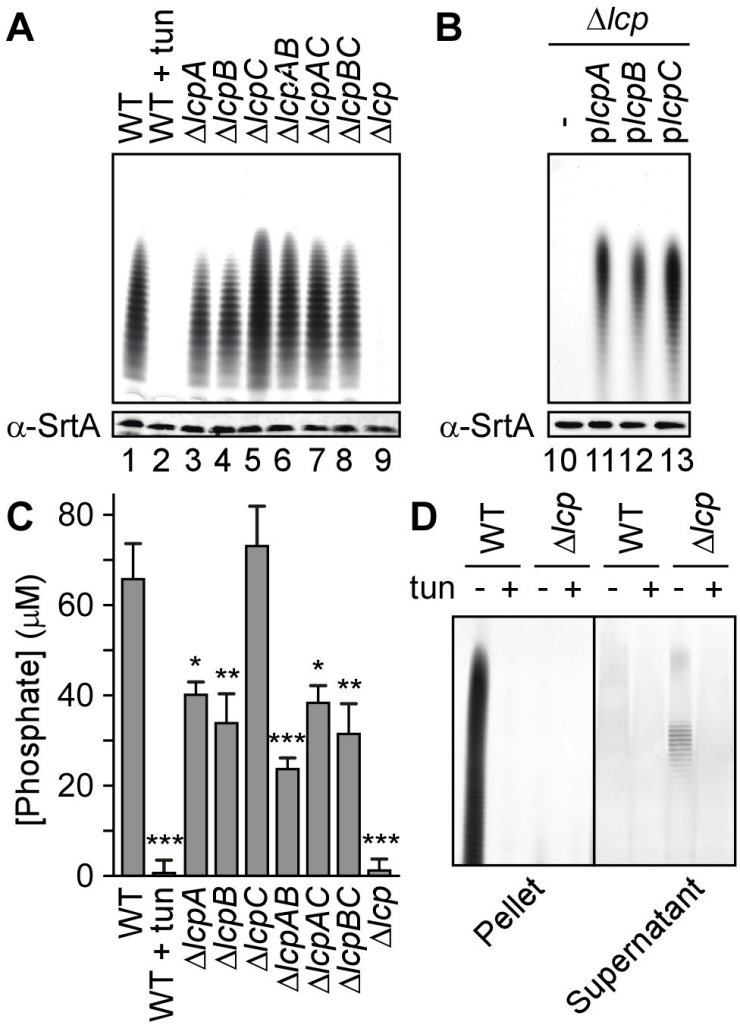

FIG. 1. Δ*lcp* mutant cells lack cell-associated wall teichoic acid. (A and B) Alcian blue- and silver-stained acrylamide gels of cell wall-associated WTA. (A) WTA was extracted from *S. aureus* MSSA1112 wild-type (WT) grown without (lane 1) or with (lane 2) tunicamycin and from single (Δ*lcpA*, Δ*lcpB*, Δ*lcpC*), double (Δ*lcpAB*, Δ*lcpAC*, Δ*lcpBC*) and triple (Δ*lcp*) mutant strains (lanes 3-9). (B) WTA was extracted from the triple (Δ*lcp*) mutant strain carrying an empty vector (panel B, lane 10) or complementing plasmids p*lcpA*, p*lcpB*, or p*lcpC* (panel B, lanes 11-13). As a control for the number of staphylococci, cell extracts were immunoblotted with antibodies specific for sortase A. (C) Quantification of phosphate levels in purified peptidoglycan from strains described in A. Average phosphate levels were derived from three independent experiments and the standard error of the means calculated. Statistical significance between the WT and each group was determined using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test: symbols denote * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. (D) Identification of teichoic acid in the supernatant of Δ*lcp* cultures. WT and Δ*lcp* strains were grown in the absence (-) or presence (+) of tunicamycin, and teichoic acids in cell pellets (left panel) or culture supernatants (right panel) were resolved by PAGE and visualized by staining with alcian blue and silver.

Figure 2

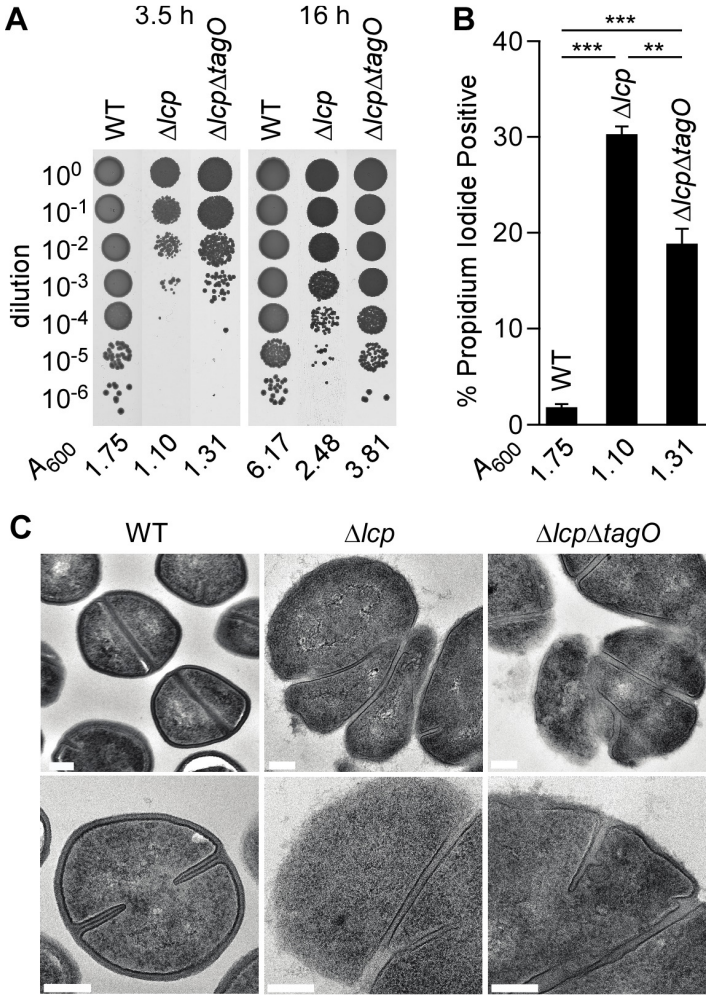

FIG. 2. Deletion of *tagO* does not improve growth of the Δ*lcp* mutant. (A) Staphylococcal viability was examined by plating serial dilutions (0- to 6-fold) of culture aliquots of WT, Δ*lcp*, and Δ*lcp*Δ*tagO* mutants that had been grown for 3.5 and 16 h, respectively. Images of the agar plates are shown along with absorbance values of cultures at 600 nm (A_{600}) at the time of plating. (B) Membrane integrity of staphylococci was assessed with propidium iodide staining. Culture aliquots of WT, Δ*lcp*, and Δ*lcp*Δ*tagO* mutants that had been grown for 3.5 h were fixed with paraformaldehyde and stained with SYTO 9 (total cells) and propidium iodide. SYTO 9-positive cells were analyzed for propidium iodide staining using flow cytometry. Combined data from two independent experiments with triplicate analyses of 20,000 cells/sample are presented. Statistical significance was determined using the Student's *t* test (* denotes $p < 0.05$). (C) Transmission electron micrographs of WT, Δ*lcp*, and Δ*lcp*Δ*tagO* bacteria. Cells were cultured for 3.5 h as in panel A. Scale bars represent 200 nm.

Figure 3

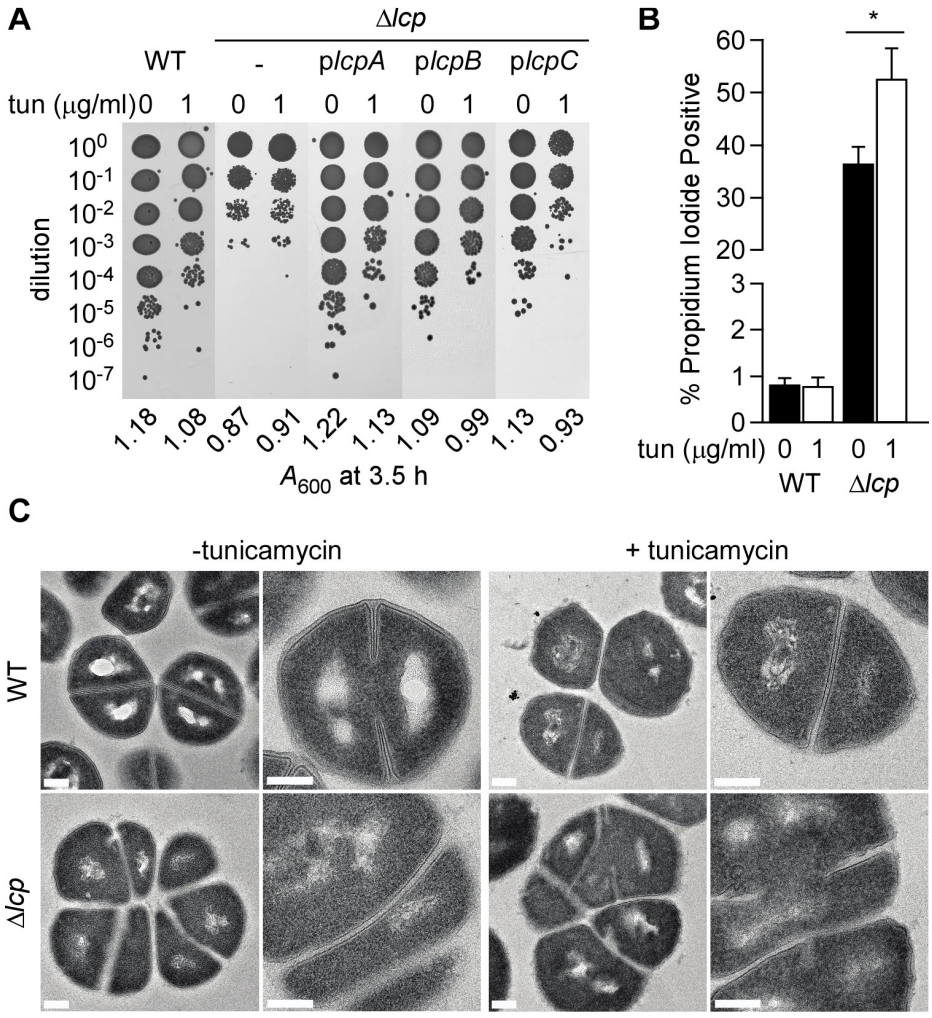

FIG. 3. Inhibition of TagO with tunicamycin does not suppress the growth defects of the Δ*lcp* mutant. (A) Bacterial growth was examined by plating serial dilutions of cultures grown for 3.5 h without or with tunicamycin (0 or 1 μg/ml). Culture aliquots of WT and the Δ*lcp* mutant without or with p*lcpA*, p*lcpB*, or p*lcpC* were spotted on agar plates. A_{600} values were recorded at the time of plating are reported under the images of agar plates. (B) Membrane integrity of staphylococci assessed with propidium iodide staining. Culture aliquots of WT and Δ*lcp* mutant grown for 3.5 h without or with tunicamycin (0 or 1 μg/ml) were fixed and stained as described in Fig. 2B. The data were analyzed as described in Fig. 3B (* $p < 0.05$). **C.** Transmission electron micrographs of WT and Δ*lcp* mutant cells from cultures grown for 3.5 h without or with tunicamycin (0 or 1 μg/ml). Scale bars represent 200 nm.

Figure 4

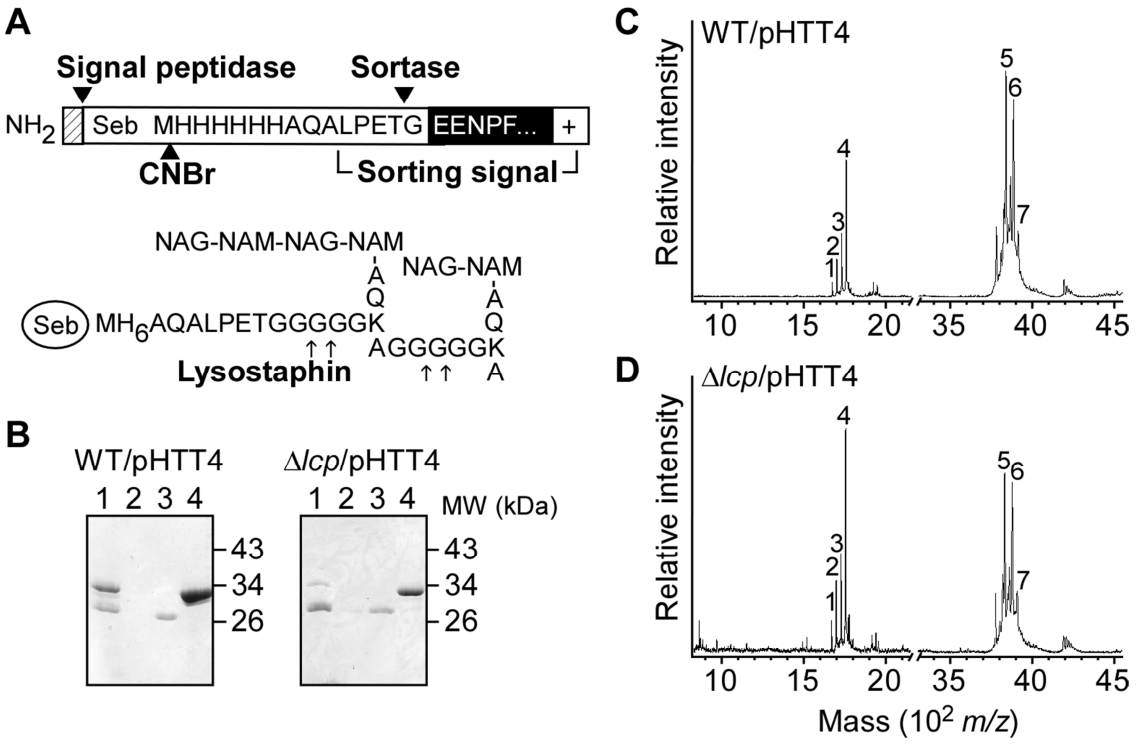

FIG. 4. Sortase A-anchoring of proteins to the cell wall is not affected in the Δ*lcp* mutant. (A) Diagram of recombinant SEB-MH_6-CWS encoded by the pHTT4 plasmid. Cleavage sites of signal peptidase, sortase A and cyanogen bromide (CnNBr) are indicated. (B) Coomassie-Brilliant Blue-stained SDS-PAGE of SEB-MH_6-CWS released with lysostaphin from the cell wall of either wild-type (left) and Δ*lcp* mutant (right) staphylococci. Solubilized cell wall preparations (lanes 1) were subjected to affinity chromatography on Ni-NTA beads. Material not retained on the column is shown in lanes 2. The beads were washed (lanes 3) and SEB-MH_6-CWS eluted with buffer containing 0.5 M imidazole (lanes 4). Molecular weight markers (MW in kDa) are indicated on the right side of the gel. (C and D) Mass spectrometry of CNBr cleaved SEB-MH_6-CWS purified from wild-type (C) and Δ*lcp* mutant (D). Proteins eluted in lanes 4 panel B were digested with CNBr and fragments were purified once more over Ni-NTA, eluted, desalted and subjected to MALDI-TOF MS. The analysis of the ion signals labeled 1-7 and structural predictions are listed in Table 2.

Figure 5

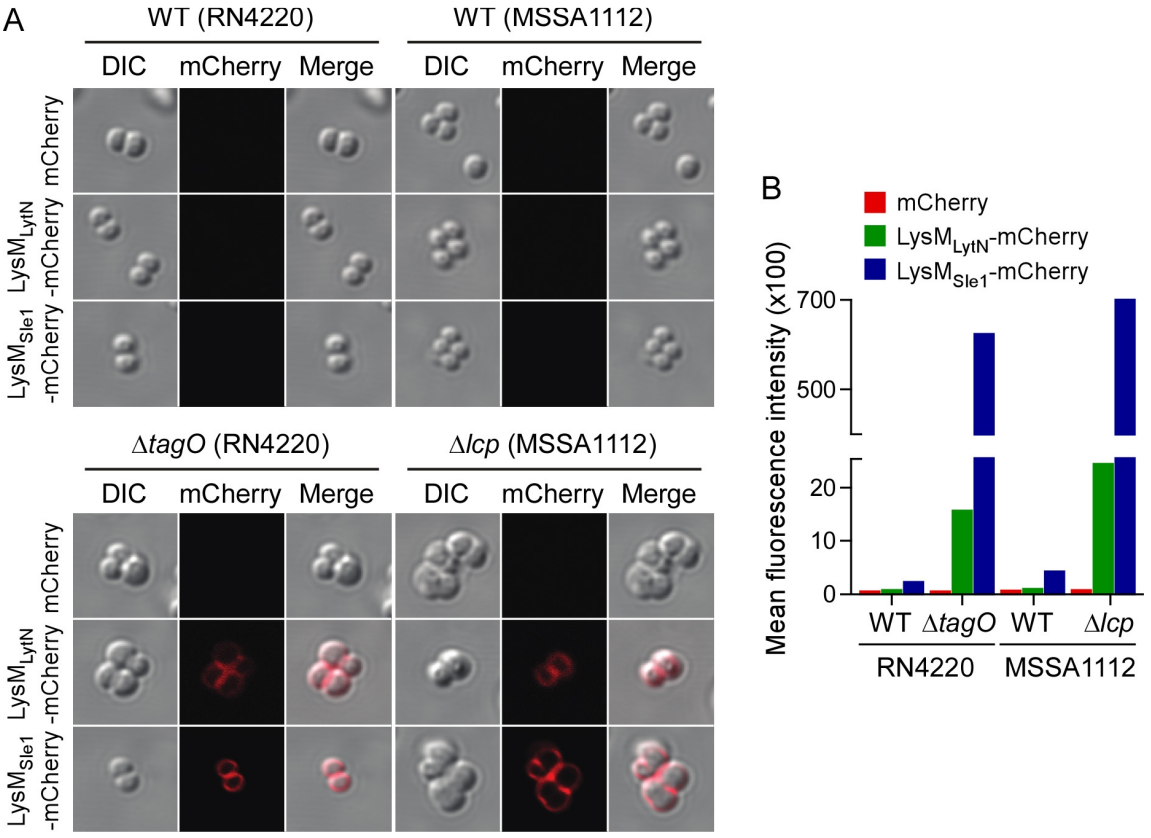

FIG. 5. The LysM domains of LytN and Sle1 bind uniformly to the envelope of the Δ*lcp* mutant. Purified mCherry, LysM$_{LytN}$-mCherry, or LysM$_{Sle1}$-mCherry were incubated with wild-type parent (strains RN4220 and MSSA1112), Δ*tagO* or Δ*lcp* staphylococci. Binding of hybrid mCherry to the bacterial envelope was analyzed by fluorescence microscopy (A) and flow cytometry (B). The left panels in (A) display the DIC image of staphylococcal cells analyzed by fluorescence microscopy (middle panels). The right panels display merged images derived from both data sets.